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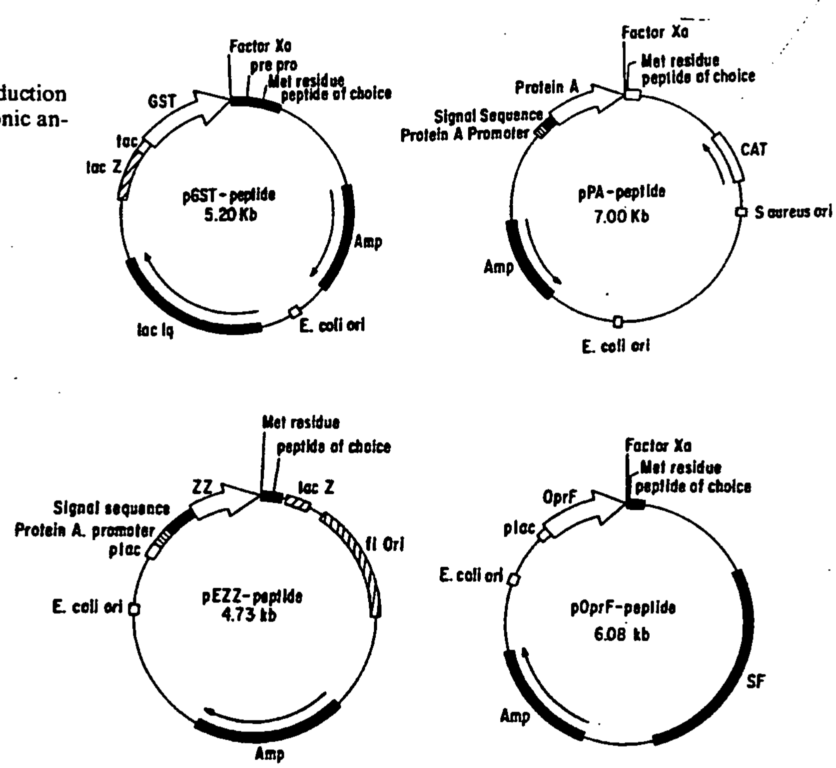
(57) Abstract

Method for recombinant production of cationic peptides and a polycationic antibacterial peptide.



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CATIONIC PEPTIDES AND METHOD FOR PRODUCTION

This application is a continuation-in-part of Application Serial No. 07/933,492, filed August 21, 1992.

BACKGROUND OF THE INVENTION

1. *Field of the Invention*

The invention relates to recombinant vector systems and more specifically to the use of these systems for production of cationic peptides.

2. *Description of Related Art*

In 1981, the self-promoted uptake hypothesis was first proposed to explain the mechanism of action of polycationic antibiotics in *Pseudomonas aeruginosa*. According to this hypothesis, polycations interact with sites on the outer membranes of Gram-negative bacteria at which divalent cations cross-bridge adjacent lipopolysaccharide molecules. Due to their higher affinity for these sites, polycations displace the divalent cations and, since the polycations are bulkier than the divalent cations, cause structural perturbations in the outer membrane. These perturbations result in increased outer membrane permeability to compounds such as the β-lactam antibiotic nitrocefin, the eukaryotic non-specific defense protein lysozyme and to hydrophobic substances. By analogy, molecules accessing this pathway are proposed to promote their own uptake.

It has been clearly demonstrated that the outer membranes of Gram-negative bacteria are semipermeable molecular "sieves" which restrict access of antibiotics and host defense molecules to their targets within the bacterial cell. Thus, cations and polycations which access the self-promoted uptake system are, by virtue of their ability to interact with and break down the outer

membrane permeability barrier, capable of increasing the susceptibility of Gram-negative pathogenic bacteria to antibiotics and host defense molecules. Hancock and Wong demonstrated that a broad range of such compounds could overcome the permeability barrier and coined the name "permeabilizers" to describe them (Hancock and Wong, *Antimicrob. Agents Chemother.*, 26:48, 1984). While self-promoted uptake and permeabilizers were first described for *P. aeruginosa*, they have now been described for a variety of Gram-negative bacteria.

Over the past decade, non-specific defense molecules have been described in many animals, including insects and humans. One subset of these molecules have in common the following features: (a) they are small peptides, usually 15-35 amino acids in length, (b) they contain 4 or more positively charged amino acid residues, either lysines or arginines, and (c) they are found in high abundance in the organisms from which they derive. Several of these molecules have been isolated, amino acid sequenced and described in the patent literature (e.g., cecropins: WO8900199, WO 8805826, WO8604356, WO 8805826; defensins: EP 193351, EP 85250, EP 162161, US 4659692, WO 8911291). However, only limited amounts of these peptides can be isolated from the host species. For example, Sawyer, *et al.*, (*Infect. Immun.* 56:693, 1988) isolated 100-200 mg of rabbit neutrophil defensins 1 and 2 from 10^9 primed peritoneal neutrophils or lipopolysaccharide-elicited alveolar macrophages (i.e., the numbers present in a whole animal).

The gene for human defensin has been cloned and sequenced, but no successful expression has been demonstrated, as yet. Furthermore, production of these peptides using peptide synthesis technology produces peptides in limited amounts and is expensive when scaled up or when many variant peptides must be produced. Also, structural analysis is difficult without

specific incorporation of ^{15}N and ^{13}C tagged amino acids which is prohibitively expensive using amino acid synthesis technology.

Therefore, a method for producing small cationic peptides, especially polycationic polypeptides, in commercially practicable amounts is needed. This invention addresses such a need by disclosing such a method using recombinant DNA technology in *Escherichia coli* and *Staphylococcus aureus*. This process has not been achieved in bacteria until the present invention, due to such difficulties as the susceptibility of these polycationic peptides to bacterial protease degradation.

SUMMARY OF THE INVENTION

This invention provides a novel method for producing a cationic peptide by recombinantly producing a fusion peptide which is composed of the cationic peptide and an anionic peptide. The fusion peptide can preferably later be cleaved to release the cationic peptide, which can then be purified. Numerous polycationic peptide structures from nature have been reported and have been either demonstrated or hypothesized to act as outer membrane permeabilizers. The cationic peptides produced by the method of the invention can be used synergistically with classical antibiotics to break down the outer membrane permeability barrier, which is one of the major limiting factors in antibiotic activity against Gram-negative bacteria. Such permeabilizers demonstrate an enormous range of amino acid sequences, lengths, and predicted 3-dimensional structures. Until the present invention, no general method had been described that would allow the production of polycationic peptides in meaningful quantities in bacteria.

In another embodiment, the invention provides a novel cationic peptide which is a fusion between selected cecropin and melittin sequences with additional modifications. This novel peptide possesses enhanced anti-bacterial activity as compared with previously described cationic peptides.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a schematic representation of the plasmids used in the invention; A, pGST-peptide; B, pPA-peptide; C, pEZZ-peptide; D, pOprF-peptide.

FIGURE 2 shows a schematic representation of constructs used in fusion protein vectors.

FIGURE 3 shows the amino acid sequences of human neutrophil peptide-1 (HNP-1), the cecropin A/melittin hybrid peptide (CEME) and (CEMA). Positively charged amino acids are in bold.

FIGURE 4 shows a killing curve for *P. aeruginosa* treated with CEME.

FIGURE 5 shows a lysozyme lysis assay for *P. aeruginosa* treated with polymyxin B (Px), CEME, CEMA, or melittin.

FIGURE 6 shows a lysozme lysis assay for *E. cloacae* treated with polymyxin B (Px), CEME, CEMA, or melittin.

FIGURE 7 shows 1-N-phenylnaphthylamine (NPN) uptake in *P. aeruginosa* after treatment with polymyxin B (Px), CEME, CEMA, or melittin.

FIGURE 8 shows 1-N-phenylnaphthylamine (NPN) uptake in *E. cloacae* after treatment with polymyxin B (Px), CEME, CEMA, or melittin.

FIGURE 9 shows inhibition of dansyl polymyxin (DPx) binding to *P. aeruginosa* lipopolysaccharide (LPS) by polymyxin B (Px), gentamycin (Gm), CEME, CEMA, or melittin.

FIGURE 10 is a double reciprocal plot of the results shown in FIGURE 9.

FIGURE 11 shows inhibition of DPx to intact *P. aeruginosa* cells in the presence of Px, Gm, CEME, CEMA, or melittin.

FIGURE 12 is a double reciprocal plot of the results shown in FIGURE 11.

DETAILED DESCRIPTION OF THE INVENTION

The method of this invention provides production of fusion proteins comprising an anionic peptide and a cationic peptide. Initially, when sequences encoding cationic peptides were placed into expression vectors without an amino terminal sequence encoding an anionic carrier peptide or with just a signal sequence encoding region or pre- pro defensin encoding region (i.e., the regions which result in export in bacteria or eukaryotes, respectively), no peptide was observed despite the presence of measurable mRNA levels in the cytoplasm. This finding indicated that some intracytoplasmic mechanism might somehow be preventing the translation of the mRNA or degrading the translation product. This problem has been solved in the present invention by fusing an anionic carrier peptide to the cationic peptide such that stabilization occurs and breakdown by bacterial proteases is avoided.

As used herein, the term "cationic peptide" refers to a sequence of amino acids from about 5 to about 50 amino acids in length and preferably from about 15 to about 35 amino acids in length. A peptide is "cationic" if it possesses sufficient positively charged amino acids that has a pKa greater than 9.0. Typically, at least four of the amino acid residues of the cationic peptide can be positively charged, for example, lysine or arginine. "Positively charged" refers to the side chains of the amino acid residues which have a net positive charge at pH 7.0. Examples of naturally occurring cationic peptides which can be recombinantly produced according to the invention include defensins, magainins, melittin, and cecropins, and analogs thereof.

According to the method of the present invention, the fusion peptide comprises a "carrier peptide" which is encoded by a nucleotide sequence present in the expression vector. The "carrier peptide" is preferably located at the amino-terminal end of the fusion peptide sequence. The carrier peptide is sufficiently

anionic such that the positive charge associated with the cationic peptide is overcome. As a result, the fusion peptide has a net charge which is essentially neutral or even negative. The anionic carrier peptide may be derived from naturally-occurring proteins or can be entirely artificial. Functionally, the carrier peptide stabilizes the cationic peptide and protects it from bacterial proteases.

The carrier peptide of the method of the invention may further function to transport the fusion peptide to inclusion bodies, the periplasm, the outer membrane or, preferably, the external environment. Categories of carrier peptide which can be utilized according to the invention include anionic pre-pro peptides and anionic outer membrane peptides. Carrier peptides of the invention specifically include, but are not limited to glutathione-S-transferase (GST) (Smith *et al., Proc. Natl. Acad. Sci. USA*, 83:8703 (1986)), protein A from *Staphylococcus aureus* (Nilsson, *et al., EMBO* 4:1075 (1985)), two synthetic IgG-binding domains (ZZ) of protein A (Löwenadler, *et al., Gene*, 58:87, 1987) and outer membrane protein F from *Pseudomonas aeruginosa* (Duchene, *et al., J. Bacteriol*, 170:155, 1988). The invention is not limited to the use of these peptides as carriers; others with similar properties as the carriers described herein are known to those skilled in the art, or can be readily ascertained without undue experimentation.

Techniques for the isolation and purification of the microbially expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibody. The use of glutathione-S-transferase (GST) as the carrier protein, allows purification with a glutathione agarose affinity column. When either Protein A or the ZZ domain from *Staphylococcus aureus* is used as the carrier protein, single step purification can be done using an IgG-sepharose affinity column, for example. Use of pOprF-peptide, the N-terminal half of the *P.*

aeruginosa outer membrane protein F, is advantageous, since it is the prominent protein band found in outer membrane preparations and therefore can also be purified easily. Alternatively, the fusion peptides can be isolated by use of reagents specifically reactive with the cationic peptide portion of the fusion peptide. Along these lines, monoclonal antibodies which bind an epitope present in the cationic peptide can be utilized, for example, by using standard solid phase affinity purification methodology.

In one embodiment of the invention, a signal sequence is included in the expression vector, specifically located adjacent to the N-terminal end of the carrier protein. The signal sequence allows the fusion protein to be directed toward the membrane. The signal sequence usually consists of a leader of from about 16 to about 29 amino acids, starting with two or three polar residues and continuing with a high content of hydrophobic amino acids; there is otherwise no detectable conservation of sequence known. While the vectors used as examples in the present invention use the protein A signal sequence of *Staphylococcus* or the OprF signal sequence in *Pseudomonas aeruginosa*, other signal sequences which provide the means for transport of the fusion protein to the cell membrane will be equally effective in carrying out the method of the invention. Such signal sequences are known to those of skill in the art.

According to the invention, it may be advantageous to include a "spacer DNA sequence" in the vectors used in the method of the invention. As used herein, "spacer DNA sequence" refers to any DNA sequence located between the carrier peptide DNA sequence and the cationic peptide DNA sequence of the fusion peptide vector. While not wanting to be bound to a particular theory, it is believed that the spacer DNA sequence, when translated, may create a "hinge-like" region which allows the negatively charged residues of the anionic carrier peptide and the positively charged residues of the subject cationic

peptide to interact, thereby inhibiting the positive charge effect and associated detrimental phenomena, such as degradation by proteolytic enzymes.

In addition to stability, the spacer DNA sequence may provide a site for cleavage of the carrier peptide from the peptide after synthesis of the fusion peptide. For example, such spacer DNA sequences include, but are not limited to, protease cleavage sequences, such as that for Factor Xa protease, the methionine, tryptophan and glutamic acid codon sequences, and the pre-pro defensin sequence. Factor Xa is used for proteolytic cleavage at the Factor Xa protease cleavage sequence, while chemical cleavage by cyanogen bromide treatment releases the peptide at the methionine or related codons. In addition, the fused product can be cleaved by insertion of a codon for tryptophan (cleavable by o-iodosobenzoic acid) or glutamic acid (cleavable by *Staphylococcus* protease). Insertion of such spacer DNA sequences is not a requirement for the production of functional cationic peptides, however, specific sequences will enhance the stability of the fusion peptide. The pre-pro defensin sequence is negatively charged, therefore it is envisioned within the invention that other DNA sequences encoding negatively charged peptides could be used as spacer DNA sequences to stabilize the fusion peptide and prevent degradative events, such as bacterial proteolytic degradation. Spacer DNA sequences, such as the pre-pro defensin amino terminal sequences, are also efficient in the transport of fusion peptide to the outside of the host cell membrane.

In the present invention, the cationic sequences may be inserted into a recombinant "expression vector". The term "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of cationic genetic sequences. Such expression vectors of the invention are preferably plasmids which contain a promoter sequence which facilitates the efficient transcription of the inserted genetic

sequence in the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. For example, the expression of the fusion peptide of the invention can be placed under control of *E. coli* chromosomal DNA comprising a lactose or lac operon which mediates lactose utilization by elaborating the enzyme beta-galactosidase. The lac control system can be induced by IPTG. A plasmid can be constructed to contain the *lac Iq* repressor gene, permitting repression of the *lac* promoter until IPTG is added. Other promoter systems known in the art include beta lactamase, lambda promoters, the protein A promoter, and the tryptophan promoter systems. While these are the most commonly used, other microbial promoters, both inducible and constitutive, can be utilized as well. The vector contains a replicon site and control sequences which are derived from species compatible with the host cell. In addition, the vector may carry specific gene(s) which are capable of providing phenotypic selection in transformed cells. For example, the beta-lactamase gene confers ampicillin resistance to those transformed cells containing the vector with the beta-lactamase gene.

Transformation of the host cell with the recombinant DNA may be carried out by conventional techniques well known to those skilled in the art. For example, where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth and subsequently treated by the $CaCl_2$ method using procedures well known in the art. Alternatively, $MgCl_2$ or RbCl could be used.

In addition to conventional chemical methods of transformation, the plasmid vectors of the invention may be introduced into a host cell by physical means, such as by electroporation or microinjection. Electroporation allows transfer of the vector by high voltage electric impulse, which creates pores in the plasma membrane of the host and is performed according to methods well

known in the art. Additionally, cloned DNA can be introduced into host cells by protoplast fusion, using methods well known in the art.

DNA sequences encoding the cationic peptides can be expressed *in vivo* by DNA transfer into a suitable host cell. "Host cells" of the invention are those in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that not all progeny are identical to the parental cell, since there may be mutations that occur during replication. However, such progeny are included when the terms above are used. Preferred host cells of the invention include *E. coli*, *S. aureus* and *P. aeruginosa*, although other Gram-negative and Gram-positive organisms known in the art can be utilized as long as the expression vectors contain an origin of replication to permit expression in the host.

The cationic peptide DNA sequence used according to the method of the invention can be isolated from an organism or synthesized in the laboratory. Specific DNA sequences encoding the cationic peptide of interest can be obtained by: 1) isolation of a double-stranded DNA sequence from the genomic DNA; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the cationic peptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA.

The synthesis of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired peptide product is known. In the present invention, the synthesis of a DNA sequence has the advantage of allowing the incorporation of codons which are more likely to be recognized by a bacterial host, thereby permitting high level expression without difficulties in translation. In addition, virtually any peptide can be synthesized,

including those encoding natural cationic peptides, variants of the same, or synthetic peptides.

When the entire sequence of the desired peptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the formation of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid or phage containing cDNA libraries which are derived form reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the cationic peptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single stranded form (Jay, *et al.*, *Nuc. Acid Res.*, 11:2325, 1983).

The invention also provides an isolated peptide comprising Sequence ID No. 24, and conservative variations thereof. The peptide, CEMA, contains the first 18 amino acids of cecropin and the last 8 amino acids of melittin. CEMA has been altered by changing the carboxy terminal amino acid sequence of CEME to include two extra lysine residues. This modification unexpectedly created a sequence with two-fold improved antibiotic activity against many bacterial species as well as a substantially enhanced ability to permeabilize bacterial outer membranes and bind to lipopolysaccharide (LPS). Alternatively, other positively charged amino acids could be substituted for lysine, as long as their charge is positive at pH 7.0.

Minor modifications of the primary amino acid sequence of the peptide of the invention may result in peptides which have substantially equivalent activity as compared to the specific peptide described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the peptides produced by these modifications are included herein as long as the biological activity of the original peptide still exists. Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would also have utility. For example, amino or carboxy terminal amino acids which may not be required for biological activity of the particular peptide can be removed.

The peptide of the invention include peptides which are conservative variations of those peptides specifically exemplified herein. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

The invention also provides polynucleotides which encode the peptides of the invention. As used herein, "polynucleotide" refers to a polymer of deoxyribonucleotides or ribonucleotides, in the form of a separate fragment or as a component of a larger construct. DNA encoding a peptide of the invention can be assembled from cDNA fragments or from oligonucleotides which provide a synthetic gene which is capable of being expressed in a recombinant

transcriptional unit. Polynucleotide sequences of the invention include DNA, RNA and cDNA sequences. A polynucleotide sequence can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. Polynucleotides of the invention include sequences which are degenerate as a result of the genetic code.

The invention also provides a method of inhibiting the growth of bacteria comprising contacting the bacteria with an inhibiting effective amount of the CEMA peptide of the invention. The term "contacting" refers to exposing the bacteria to CEMA so that CEMA can effectively inhibit, kill, or lyse bacteria, bind endotoxin (LPS), or permeabilize gram- negative bacterial outer membranes. Contacting may be *in vitro*, for example by adding CEMA to a bacterial culture to test for susceptibility of the bacteria to the peptide. Contacting may be *in vivo*, for example administering CEMA to a subject with a bacterial disorder, such as septic shock. "Inhibiting" or "inhibiting effective amount" refers to the amount of CEMA which is required to cause a bacteriastatic or bacteriacidal effect. Examples of bacteria which may be inhibited include *E. coli*, *P. aeruginosa*, *E. cloacae*, *S. typhimurium*, and *S. aureus*. The method of inhibiting the growth of bacteria may further include the addition of antibiotics for combination or synergistic therapy. The appropriate antibiotic administered will typically depend on the susceptibility of the bacteria such as whether the bacteria is gram negative or gram positive, and will be easily discernable by one of skill in the art.

The peptide of the invention can be administered parenterally by injection or by gradual infusion over time. The peptide can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally. Preferred methods for delivery of the peptide include orally, by encapsulation in microspheres or proteinoids, by aerosol delivery to the lungs, or

transdermally by iontophoresis or transdermal electroporation. Other methods of administration will be known to those skilled in the art.

Preparations for parenteral administration of a peptide of the invention include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be utilized.

EXAMPLE 1

Construction of pGST-peptide Plasmids

This plasmid was derived from pGEX-3X (Smith, *et al., Proc. Natl. Acad. Sci., U.S.A.*, 83:8703, 1986) using recombinant DNA technology. A 500 bp *BclI-BamHI* fragment from pGEX-3X that included the Factor X cleavage site and part of the glutathione-S-transferase gene was isolated. This fragment was subjected to 35 cycles of PCR (94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute) in an Ericomp themocyler, using primers at both ends of the

fragment. The primer used at the end that had been cleaved with *BamHI* had a protruding 5' end which resulted in the addition of *SphI, HindIII* and *EcoRI* restriction sites after the PCR reaction. The *SphI* site was flush against the Factor X cleavage site. The resulting PCR fragment was made blunt-ended using Klenow fragment and ligated into the *SmaI* site of pTZ18U in order to obtain a stable copy. Using *BclI* and *EcoRI*, the fragment was cleaved out of pTZ18U and ligated into pGEX-3X that had been cut with *EcoRI* and partially with *BclI*. The resulting plasmid, pGEX-KP, now had a *SphI-HindIII-EcoRI* multiple cloning site, changed from *BamHI-SmaI-EcoRI* in PGEX-3X.

Cloning and Expression of GST-peptide Genes

Synthetic DNAs coding for CEME (Table 1A, B), CEMA (Table I, V, W) and HNP-1 (Table 1C-H) were synthesized as overlapping oligonucleotides which were annealed and ligated into pGEX-KP where HNP-1 is the human neutrophil peptide 1 and CEME is a fusion peptide made from portions of an insect defensin ceropin A and the bee venom peptide, melittin and CEMA is a variant of CEME with two additional lysine residues at the carboxy-terminal end (FIGURE 3). However, since these genes are wholly synthetic, virtually any peptide sequence or variant of CEME and HNP-1 can be incorporated (FIGURE 2). Positive clones were identified by slot lysis gel electrophoresis and confirmed by DNA sequencing on an Applied Biosystems automated DNA sequencer, using synthetic oligonucleotides as primers (Table 1 T-U). Strains containing the recombinant vectors were tested for fusion protein expression. Briefly, cells were grown in Luria broth to $OD_{600} \approx 1.0$. IPTG was added to 0.2mM to induce expression. Samples of uninduced and induced cells were resuspended in loading buffer and subjected to SDS polyacrylamide gel electrophoresis, revealing that the fusion protein constituted as much as 15% of total cellular protein. In the case of HNP-1, the resultant fusion protein was

unstable, due to proteolysis. Inclusion of a synthetic, pre-pro defensin-encoding DNA cassette rendered the fusion protein stable to proteolysis.

Purification and Cleavage of the GST-peptide Protein

Cells induced for the production of the fusion protein were harvested and lysed by passage through a French pressure cell (15000 psi). The lysate was fractionated by centrifugation at 3000 xg and the fusion protein was found in either the pellet (as inclusion bodies) or in the supernatant. If found in the soluble supernatent fraction, the fusion protein was then incubated with glutathione-agarose or glutathione-sepharose beads (sulfur linkage, Sigma). The beads bound the fusion protein and were washed with several volumes of buffer to remove unbound protein. The bound fusion protein was then eluted with 0.5% SDS in order to ensure complete recovery. FIGURE 5 shows the results of a typical isolation procedure. SDS removal was achieved by $CHCl_3MeOH$ (2:1) extraction or ethanol precipitation of the protein, followed by lyophilization. If found in the insoluble pellet fraction, the protein was isolated by extraction with 3% octyl-polyoxyethylene (O-POE) which removes membrane proteins which are the major contaminating species in inclusion body preparation. All detectable levels of membrane proteins are solubilized with O-POE, leaving a relatively pure sample of inclusion bodies. This sample is extracted with 8 M urea to solubilize the inclusion bodies, after which the urea is removed by dialysis. At this stage we attempted to release the peptide using Factor Xa protease cleavage, but found this to be extremely inefficient. Therefore, a synthetic methionine residue codon was placed immediately adjacent to the polycationic peptide sequence to permit chemical cleavage. In this case, after the inclusion body preparation was extracted with O-POE, the inclusion bodies were solubilized directly in 70% formic acid, to which CNBr was added in order to release the polycationic peptide from the fusion protein. In the final invention the methionine residue is always present.

TABLE 1

Sequence 5' - 3'	Description
A) CGGGGATCCGCATATGAAATGGAAACTGTTCAAGAAGA TCGGCATCGGCGCCGTGCTGAAAGTGCTGACCACCGGT CTGCCGGCGCTGATCAGCTAACTAAGTA	104 mer encoding CEME
B) AGCTTACTTAGTTAGCTGATCAGCGCCGGCAGACCGGT GGTCAGCACTTTCAGCACGGCGCCGATGCCGATCTTCTT GAACAGTTTCCATTTCATATGCGGATCCCCGCATG	112 mer encoding CEME
C) GGGAGCTCCTAACTAACTAAGGAGGAGACATATGAAAC AAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCCC	81 mer used in construction of HNP-1 gene
D) CCAGTGCAATAGTGCTTTGTTTCATATGTCTCCTCCTTA GTTAGTTAGGAGCTCC	56 mer used in construction of HNP-1 gene
E) TGTGACAAAAGCCGCATGCTACTGCCGTATACCGGCCT GCATCGCGGGCGAACGTCGTTACGGTA	65 mer used in construction of HNP-1 gene
F) CAGGCCGGTATACGGCAGTAGCATGCGGCTTTTGTCAC AGGGGTAAACAGTAACGGTAAGAGTG	64 mer used in construction of HNP-1 gene
G) CCTGCATCTACCAGGGCCCTCTGTGGGCGTTCTGCTG CTAAAAGCTTCGC	50 mer used in construction of HNP-1 gene
H) GCGAAGCTTTTAGCAGCAGAACGCCCACAGACGGCCC TGGTAGATGCAGGTACCGTAACGACGTTCGCCCGCGATG	76 mer used in construction of HNP-1 gene
I) CCATATGAGGACCCTCGCCATCCTTGCTGCCATTCTCCT GGTGGCCCTGCAGGCCCAGGCTGAGCCACTCCAGGCAA GAGCTGATGAGGTTGCAGCAGCCCCGGAGCAGA	110 mer used in construction of pre pro cartridge
J) TTGCAGCTGACATCCCAGAAGTGGTTGTTTCCCTTGCAT GGGACGAAACGTTGGCTCCAAAGCATCCAGGCTCAAGG AAAAACATGGCATG	91 mer used in construction of pre pro cartridge
K) CCATGTTTTTCCTTGAGCCTGGATGCTTTGGAGCCAAGC TTTCGTCCCATGCAAGGGAAACAACCACTTCTGGGATG TCAGCTGCAATCTGCTCCGGGGCTGCTGCAAC	109 mer used in construction of pre pro cartridge
L) CTCATCAGCTCTTGCCTGGAGTGGCTCAGCCTGGGCCTG CAGGGCCAGCAGGAGAATGGCAGCAAGGATGGCGAGG GTCCTCATATGGCATG	92 mer used in construction of pre pro cartridge

Sequence 5' → 3'	Description
M) AGCTTGTCGACA	12 mer encoding a *HindIII* to *SalI* adaptor
N) CGTCGACATCGAAGGTCGTGCATG	24 mer encoding factor X_a recognition site and an *SphI* to *SalI* adaptor
O) CACGACCTTCGATGTCGACGCATG	24 mer encoding factor X recognition site and an *SphI* to *SalI* adaptor
P) AATTCGGATCCG	12 mer encoding an *EcoR1* to *BamHI* adaptor
Q) CGGATCCATGGCATG	15 mer encoding a methionine residue and an *SphI* to *BamHI* adaptor
R) CCATGGATCCGCATG	15 mer encoding a methionine residue and an *SphI* to *BamHI* adaptor
S) TATGGGATCCCA	12 mer encoding an *NdeI* to *BamHI* adaptor
T) CCAAAATCGGATCTGATCGAAGG	23 mer used as sequencing primer
U) CAGATCGTCAGTCAGTCACG	20 mer used as sequencing primer
V) GGCGCTGAAGCTAACTAAGTAAGCTTG	27 mer encoding CEMA
W) AATTCAAGCTTACTTAGTTAGCTTCAGCGCC	31 mer encoding CEMA

EXAMPLE 2

Construction of pPA Vectors Containing CEME and HNP-1

Using a cloned copy of the CEME gene (FIGURE 2A), oligonucleotides N + O (Table 1) were annealed and cloned into the *SphI* site at the 5' end of the gene (FIGURE 2A). The orientation was confirmed by DNA sequencing to ensure that the *SalI* site was 5' to the Factor Xa recognition site. Using oligonucleotide M (Table 1), a *SalI* site was inserted into the 3' *HindIII* site. The resulting construct (FIGURE 2B) had a *SalI* cassette that could be cloned into the *SalI* site of pRIT5, producing pPA-CEME (FIGURE 1B). The orientation of the insert was ascertained using asymmetric restriction endonuclease sites. A cloned copy of the CEMA gene was cloned into the pPA vector as described for CEME.

The HNP-1 gene (FIGURE 2C) was altered in a similar manner using oligonucleotides Q + R (for insertion into the 5' *SphI* site) and oligonucleotide P (for insertion into the 3' *EcoRI* site). The resulting construct (FIGURE 2D) had a *BamHI* cassette that could be cloned into the *BamHI* site of pRIT5 to give pPA-HNP1. In order to examine the effects of the pre-pro cartridge in this fusion protein system, the following construct had to be made. Using the *SphI* sites, the pre-pro cassette (FIGURE 2E) was inserted into the 5' end of the HNP-1 gene (FIGURE 2C). The 3' end of this construct was changed using oligonucleotide P (Table 1) and the 5' end was altered using oligonucleotide S (Table 1). The resulting construct (FIGURE 2F) was cloned into pPA-peptide using *BamHI* to produce pPA-proHNP1. Again, symmetric restriction endonuclease sites were used to confirm the orientation of the DNA fragment.

Production and Purification of Fusion Proteins From pPA-peptide

The plasmid pPA-CEME was transformed into *E. coli* DH5α; however, expression attempts in this strain revealed that the heterologous protein was being proteolytically degraded. Therefore, the plasmid was transferred to *S. aureus* strain RN4220, a restriction modification mutant, (a gift from S. Kahn) using electroporation. Cells were grown in LB media supplemented with 10 μg/ml chloramphenicol to $OD_{600} \approx 1.0$ at which time they were harvested. Culture supernatent was adjusted to pH 7.6 with NaOH and passed over an IgG Sepharose column (Pharmacia) previously equilibrated with TST (50 mM Tris-HCL pH 7.6, 150 mM NaC1 + 0.05% Tween 20) buffer. The column was washed sequentially with 10 volumes of TST and 5 volumes of 15 mM NH_4Ac pH 5.0. Finally, the protein was eluted with 0.5 M HAc pH 3.4 and directly lyophilized, to give quite a pure preparation. We have also shown that the synthetic gene for HNP-1 (with and without the pre pro cartridge) can be expressed successfully in this system with virtually no proteolytic degradation.

Cleavage of the Fusion Protein and Purification of CEME

The lyophilized heterologous protein isolated on the IgG Sepharose column was resuspended in 70% formic acid containing 1M CNBr, and the reaction was allowed to proceed for 18 hrs at 25°C in the dark. The reaction was quenched by diluting the sample to 5% formic acid and lyophilizing it. The sample was resuspended in 0.1% trifluoroacetic acid, loaded onto a ProRPC FPLC column (Pharmacia) and eluted with a 0-40% gradient of acetonitrile + T 0.1% TFA. The CEME peptide eluted between 30-35% resulting in a partially purified sample.

Antibacterial Activity of CEME

A sample of the partially purified CEME protein was electrophoresed on a 15% acid-urea gel, which separates proteins based on charge and mass (Panyim and Chalkey, *Arch. Biochem. Biophys.*, 130:337, 1969). The pH of the buffer is acidic (≈ pH 5.0) and the polarity is reversed such that proteins migrate to the cathode. Therefore, the small cationic peptides run relatively quickly through the gel and thus can be easily identified. Antibacterial activity was tested as previously described (Hultmark, *Eur. J. Biochem.*, 106:7, 1980). The gel was incubated in Mueller Hinton broth + 0.2M $NaPO_4$ pH 7.4 for 1 hr. It was overlaid with 5 mls of the same media containing 0.6% agar and ≈ 10^5 *E. coli* strain DC2, and then again with 5 ml of the same media containing only 0.6% agar. The overlaid gel was incubated overnight at 37°C which resulted in zones of clearing corresponding to the migration site of melittin (positive control) and the CEME produced in this study. Melittin is used as a positive control due to its antibacterial activity and similarity in size and PI values.

The antimicrobial activity of CEME was also quantitated using a killing assay in which the peptide was incubated with 10^6 cells/ml of *P. aeruginosa* for 20-60 min before the cells were plated for viability (FIGURE 4). The results showed that 2.5 μg/ml (0.9 μM) of CEME reduced the viable count of *P. aeruginosa* by three log orders in 20 minutes.

The minimum inhibitory concentrations of (MIC) of CEME, CEMA and melittin were determined for a number of different bacteria (Table 2). Briefly, cells were grown overnight at 37°C in LB-S (Luria broth without any salt supplement) and diluted one in 10,000 in the same medium to give concentrations of about 10^4 to 10^5 CFU/ml. The broth dilutions were set up in a 96 well microtiter plate by putting 200μl of LB-S containing the initial concentration of antibiotic or compound in column 1 and 100 μl of the same medium in columns 2-12. The

compounds were diluted by taking 100 μl of broth from column 1 and mixing it with column 2, resulting in a one in two dilution. This was continued to column 10. Finally, 10 μl of bacteria were pipetted into columns 1-11, and the plates incubated overnight at 37°C. The next day the plates were scored for growth in the wells, and the MIC determined.

Generally, CEME and CEMA had similar MIC values which were consistently lower than melittin. The MICs of the peptides were usually higher than those of polymyxin B, gentamicin (an aminoglycoside) and ceftazidime (a β-lactam). Both antibiotic sensitive strains (H188 and DC2) were two- to four-fold more sensitive to the cationic peptides as compared to their parental strains. Surprisingly, the mutant SC9252 which showed an increased resistance to polymyxin B, ceftazidime, and to a lesser extent gentamicin, was not resistant to the cationic peptides. The *S. typhimurium* defensin sensitive strain (C590) was also four-fold more sensitive to CEME, CEMA and polymyxin B, and two-fold more sensitive to gentamicin and melittin. The fact that the MIC of the β-lactam ceftazidime was unchanged in this strain suggested that the mutation may be affecting the self-promoted uptake pathway, possibly by a change in the sites of initial antibiotic contact on the surface of the outer membrane. The cationic peptides were also equally active against an *E. cloacae* clinical isolate strain (218S) and its β-lactam resistant mutant (218R1, a β-lactamase overproducer). The MIC values of the peptides were slightly higher for *S. aureus* but given their much higher molecular weight they are in fact more active than most compounds on a molar basis.

TABLE 2

MIC VALUES OF VARIOUS ANTIMICROBIAL AGENTS

Bacterium	Strain	Relevant Phenotype	MIC (ug/ml)*					
			PX	GM	CEF	CEME	CEMA	MEL
P. aeruginosa	H309	Wildtype	0.5	1	1	2.4	2.8	8
	H187	Parent of H188	0.5	1	2	4.8	2.8	8
	H188	Antibiotic sensitive	0.06	0.25	0.03	1.2	1.4	8
E. coli	UB1005	Parent of DC2	0.5	1	0.5	2.4	2.8	8
	DC2	Polymyxin sensitive	0.06	0.5	0.06	0.6	0.7	4
	SC9251	Parent of SC9252	0.06	2	0.12	1.2	1.4	8
	SC9252	Polymyxin resistant	4	4	0.5	1.2	1.4	8
S. typhimurium	C587	Parent of C590	1	4	0.25	2.4	5.6	16
	C590	Defensin sensitive	0.25	2	0.25	0.6	1.4	8
E. cloacae	218S	Parent of 218R1	0.5	0.5	0.5	2.4	2.8	8
	218R1	β-lactam resistant	0.5	0.5	>16	2..4	1.4	8
S. aureus	RN4220	Methicillin sensitive	>8	2	8	9.6	>5.6	8
	SAP0017	Methicillin resistant	>8	>8	>16	9.6	>5.6	8

* PX, polymyxin B; GM, gentamicin; CEF, ceftazidime; MEL, melittin.

Since divalent cations are known to stabilize the outer membranes of Gram negative bacteria by bridging adjacent lipopolysaccharide (LPS) molecules, they are able to increase the MIC values of compounds that interact with the LPS. The MIC assay was repeated in the presence of 5 mM Mg^{++} and 80 mM Na^{+} to determine whether or not this effect applied to the cationic peptides (Table 3). The results showed that the antibacterial activities of all three peptides were dramatically inhibited by the presence of Mg^{++} and only minimally inhibited by Na^{+}. These data are consistent with the hypothesis that the initial step in the antibacterial mechanism of cationic peptides is an association with the negatively charged sites on LPS molecules.

TABLE 3

EFFECT OF DIVALENT AND MONOVALENT CATIONS ON THE MICs OF CATIONIC PEPTIDES AGAINST *P. AERUGINOSA* H309

Compound	MIC (ug/ml)		
	No addition	+5 mM Mg^{++}	+ 80 mM Na^{+}
Polymyxin	0.5	1	0.5
Gentamicin	1	4	1
Ceftazidime	1	2	2
CEME	2.4	38.4	4.8
CEMA	2.8	22.4	5.6
Melittin	8	>64	16

The ability of the cationic peptides to work synergystically was examined by determining the MIC values for some commonly used antibiotics in the presence of sub-MIC levels of cationic peptides (Table 4). Generally, the peptides had very little effect on the MIC of antibiotics that are proposed to be taken up through porins (ceftazidime, imipenem, and tetracycline), although CEMA at 1/2 MIC levels reduced their MICs 2-fold. This may be due to the high membrane permeabilizing activity of CEMA (see below). However, the peptides did lower the MIC of polymyxin B, possibly due to the fact that they are all taken up by the same pathway and are therefore aiding each other's uptake. Of interest is the influence that melittin has on the MIC of polymyxin B, given the weak membrane permeabilizing activity of melittin. This may suggest that polymyxin B is disrupting the membrane, thus allowing melittin access to its target site rather than vice versa. The fact that this MIC is lower than those in the presence of CEME and CEMA may suggest that melittin is

much more active at its target site than the other two peptides, and that the rate limiting step in its antibacterial activity is its transport across the outer membrane.

TABLE 4

EFFECTS OF SUB-MIC LEVELS OF CATIONIC PEPTIDES ON THE MICs OF COMMON ANTIBIOTICS

Compound	MIC (μg/ml) in the presence of						
	No peptide	CEME	(μg/ml)	CEMA	(μg/ml)	Melittin	(μg/ml)
		1.2	0.6	1.4	0.7	4	2
Polymyxin	1	0.25	0.5	0.12	0.5	0.06	0.5
Ceftaxidime	4	4	4	2	2	2	4
Imipenem	4	4	4	2	4	4	4
Tetracycline	8	8	8	4	8	8	8

EXAMPLE 3

Construction of pEZZ-peptide Plasmids

The synthetic genes for the peptides CEME and CEMA were cloned separately into the *BamHI-HindIII* sites of the plasmid pEZZ18 (Pharmacia) (FIGURE 1C). This allowed directional insertion to occur. Clones were selected using blue/white selection by growth on IPTG - XGal media in a *lac-E. coli* strain. Correct clones were identified by restriction analysis of plasmid DNA, which was then transformed into the *E. coli* strain HB101. This strain was recommended by Pharmacia, and has been shown previously (Löwenadler, *et al.*, *Gene*, 58:87, 1987), to allow the release of fusion protein into the external medium.

Cells harboring the clones were grown to log-phase and harvested. Whole cell lysates were electrophoresed on an SDS polyacrylamide gel, and the proteins were transferred to nitrocellulose. Using antisera specific to the peptides, the fusion proteins were identified.

Construction of pOprF-peptide Plasmid

The *SalI* fragment used in construction of the pPA-CEME, was utilized here. pOprF DNA (FIGURE 1D) was isolated and digested with *SalI* to produce a linear fragment, into which the CEME cassette was ligated. Clones were analyzed at the DNA level to check for the correct orientation of the CEME DNA, using asymmetric restriction endonuclease sites. Analysis was then carried out at the protein level.

Cells were grown in selection media containing 1M IPTG. Whole cell lysates were analyzed by SDS-PAGE, and subjected to western blotting. Antisera used were (a) a polyclonal serum specific for CEME, and (b) a mAb for OprF. Results indicated both epitopes are expressed on the same molecule.

EXAMPLE 4

MEMBRANE PERMEABILIZING ACTIVITY

Membrane permeabilization by polycationic compounds was measured in two different assays. The first assay measured membrane disruption by the ability of lysozyme to reach the periplasm and cause cell lysis. This assay was performed on two different organisms in order to demonstrate that it is a general phenomenon. The uptake of lysozyme into whole cells due to membrane permeabilization by various compounds was previously described in Hancock, *et al., Antimicrobial Agents and Chemo.*, 19:777, 1981. Overnight cultures of *P. aeruginosa* H309 or *E. cloacae* 218R1 grown in LB-S were diluted 1 in 50 in fresh medium and grown to an OD_{600} = 0.5-0.6. The cells were harvested in a Silencer H-103N clinical centrifuge at 1800 g for 10 min, washed once with one volume of assay buffer (5 mM HEPES pH 7.2, 5 mM KCN), and resuspended in the same buffer to an OD_{600} of 0.5. Assays consisted of 600 μl of cells with 50 μg/ml of chicken egg white lysozyme and varying concentrations of cationic compounds. Cell lysis was measured as a decrease in the OD_{600} in a Perkin-Elmer dual beam spectrophotometer. Parallel experiments performed without lysozyme enabled the measurement of the lytic activity of the compounds themselves. To test whether or not permeability to lysozyme could be inhibited by divalent cations, various concentrations of $MgCl_2$ were added to the assay after the addition of lysozyme and before the addition of the test compound.

The results show that CEMA was a stronger permeabilizer for lysozyme than polymyxin B, as was CEME, albeit only at lower concentrations (FIGURES 5 and 6). With increased concentrations of peptide, cell lysis in the absence of lysozyme occurred, especially with CEME and melittin (*P. aeruginosa* data only). When this was taken into consideration, melittin did not appear to be a good permeabilizer as compared to the other peptides, yet it is still 5- to 10-fold better than gentamicin.

The second assay determined membrane permeability by the ability of 1-N-phenylnaphthylamine (NPN), a small fluorescent probe, to insert itself into the membrane. Normally, very little NPN can penetrate the outer membrane, but in the presence of a membrane permeabilizer it can insert into the hydrophobic membrane resulting in an increase in fluorescence (FIGURES 7 and 8). This N-phenylnaphthylamine uptake assay was previously described by Loh, *et al.*, *Antimicrobial Agents and Chemo.*, 26:546, 1984. Cells were prepared exactly the same as for the lysozyme lysis assay. 1-N-phenylnaphthylamine (NPN) was dissolved in acetone at a concentration of 500 μM. NPN fluorescence was measured in a Perkin-Elmer 650-10S fluorescent spectrophotometer using excitation and emission wavelengths set to 350 nm and 420 nm respectively, with slit widths of 5 nm. The assay was standardized by adding 20 μl of NPN (final concentration of 10 μM) and 10μl of 0.64 mg/ml of polymyxin B (final concentration of 6.4 μg/ml) into 1ml of cells, and adjusting the resulting fluorescence to read 90% deflection (90 arbitrary units). Various compounds were tested by adding 10 μl of different concentrations to a cuvette containing 1 ml of cells and 10 μM NPN. Permeabilizing activity was designated as the total fluorescence minus the fluorescence due to NPN alone. Following the fluorescence measurement, the OD_{600} of the cells was taken to ensure no significant cell lysis had occurred. Control experiments showed that neither acetone nor test compound alone resulted in an increase in fluorescence in the absence of NPN.

In these experiments, polymyxin B and CEMA were found to be equally active in opening up the membrane to NPN, while CEME and melittin (for *P. aeruginosa*) were less active. In summary, the cationic peptides were found to be strong permeabilizers of the outer membrane, which undoubtedly contributes to their mechanism of antibacterial activity.

EXAMPLE 5

INTERACTION OF CATIONIC PEPTIDES WITH LPS

The MIC assays in the presence of divalent cations indicated that the cationic peptides associated with the negatively charged sites on the LPS. To further demonstrate this quantitatively, dansyl polymyxin (DPx) displacement assays were performed using purified *P. aeruginosa* LPS. DPx is a derivative of polymyxin that fluoresces strongly when bound to LPS, but weakly when free in solution. In this assay, DPx was incubated with LPS to saturate the binding sites and then, while polycationic compounds are titrated into the reaction, displacement of the DPx was monitored by the decrease in fluorescence (FIGURE 9). *P. aeruginosa* LPS was isolated as previously described (Darveau, *et al., J. of Bacteriology,* 155:831, 1983) Dansyl polymyxin was synthesized as described by Schindler, *et al., B. Antimicrobial Agents and Chemo*, 8:95, 1975.

Briefly, 40 mgs of polymyxin B and 10 mgs of dansyl chloride were mixed in 2 mls of 60 mM $NaHC0_3$ and 40% acetone and incubated in the dark for 90 minutes. The unreacted dansyl chloride was separated from the dansyl polymyxin by gel filtration on a Sephadex G-50 column. The fractions containing dansyl polymyxin were extracted with 1/2 volume of n-butanol and then evaporated to dryness in a dessicator at 37°C. The dansyl polymyxin

was resuspended in 5 mM Hepes pH 7.0, quantitated by dinitrophenylation and stored in aliquots at -20°C.

The method of Moore, *et al.*, (*Antimicrobial Agents and Chemo.*, 29:496, 1987), was used to test how much DPx was needed to saturate the binding sties on LPS. Briefly, 5 μl samples of 100 μM DPx were titrated sequentially into 1ml of 3 μg/ml of LPS until a maximum fluorescence was reached. The fluorescence was measured in a Perkin-Elmer 650-10S fluorescence spectrophotometer with an excitation wavelength of 340 nm and an emission wavelength of 485 nm using slit widths of 5 nm. Final concentration of DPx giving 90-100% maximum fluorescence (2.5 μM) was chosen and used in all subsequent experiments. For the binding inhibition assays (Moore, *et al., Antimicrobial Agents and Chemo.*, 29:496, 1987), 2.5 μM DPx was added to 3 μg/ml of H103 LPS in 5 mM HEPES pH 7.2. The test compounds were added 5 μl at a time and the decrease in fluorescence due to displacement of the DPx from the LPS was recorded. The addition of the compound was continued until it resulted in only a small (<5%) decrease in fluorescence. The data was plotted as 1-{(maximum fluorescence-test fluorescence)/maximum fluorescence} versus the compound concentration (I). To determine the relative affinities of the compounds for the binding sites on LPS, a double reciprocal plot was made, plotting 1/(maximum fluorescence-test fluorescence)/maximum fluorescence versus 1/I. The calculated x-intercept was equivalent to $-1/I_{50}$, where I_{50} equals the concentration of the compound which displaces 50% of the DPx bound to LPS. All experiments were performed a minimum of three times.

For DPx binding inhibition assays using whole cells instead of purified LPS, H309 cells were prepared the same way as for the lysozyme lysis assay. The assay consisted of 10 μl of cells at an OD_{600} of 0.5, 990 μl of 5 mM HEPES pH 7.2 and 5 mM KCN, and the concentration of DPx that gave 90-100% binding saturation. This concentration varied from day to day but usually was between

2.5 μM and 3.5 μM. Compounds were titrated in, and I_{50} values were determined as described above.

The data showed that the cationic peptides displaced DPx at concentrations comparable to that of polymyxin B but much higher than that of gentamicin, thus indicating a strong affinity for the LPS. When the data was plotted as a double reciprocal plot (FIGURE 10), the X-intercept provided a value that is equal to $-1/I_{50}$. I_{50} is a measure of the affinity of a compound for LPS and is equal to the concentration of compound at which 50% maximal DPx displacement occurred (Table 5). The data indicated that CEMA had a higher affinity for LPS than polymyxin B (on a μM basis) with CEME and melittin having slightly lower affinities.

TABLE 5

I_{50} VALUES FOR VARIOUS COMPOUNDS AGAINST *P. aeruginosa* LPS AND WHOLE CELLS[a]

Compound	*P. aeruginosa* H103 LPS		*P. aeruginosa* H309 Whole Cells	
	Value in μM	Value in μg/ml	Value in μM	Value in μg/ml
Polymyxin	1.51 ± 0.04	2.97 ± 0.07	2.04 ± 0.65	3.7 ± 1.20
Gentamicin	19.25 ± 7.18	18.16 ± 6.77	39.13 ± 9.67	36.92 ± 9.12
$MgCl_2$-$6H_20$	1293 ± 270	262 ± 54.8	203 ± 101.00	41.2 ± 20.50
CEME	3.52 ± 1.45	9.51 ± 3.91	0.78 ± 0.04	2.11 ± 0.11
CEMA	1.32 ± 0.117	4.12 ± 0.36	0.37 ± 0.03	1.15 ± 0.09
Melittin	2.97 ± 0.506	8.48 ± 1.44	0.48 ± 0.08	1.37 ± 0.22

[a] Values are an average of at least three trials.

To determine whether or not these affinities are biologically relevant, the experiments were repeated with intact *P. aeruginosa* cells (FIGURES 11 and 12). The I_{50} values from these experiments (Table 5) showed that all three cationic peptides were better at displacing DPx from whole cells than polymyxin B. This is interesting given that only CEMA (and CEME at low concentrations) was shown to be a better membrane permeabilizer than polymyxin B. The difference between these LPS binding affinities and subsequent membrane permeabilizing activities may be indicative of the level of competing divalent cations or the conformation that the peptides adopt once they interact with the LPS.

SEQUENCE I.D. LISTING

SEQUENCE I.D. NO. 1 is A, Table 1, a primer for CEME.

SEQUENCE I.D. NO. 2 is B, Table 1, a primer for CEME.

SEQUENCE I.D. NO. 3 is C, Table 1, a primer for HNP-1.

SEQUENCE I.D. NO. 4 is D, Table 1, a primer for HNP-1.

SEQUENCE I.D. NO. 5 is E, Table 1, a primer for HNP-1.

SEQUENCE I.D. NO. 6 is F, Table 1, a primer for HNP-1.

SEQUENCE I.D. NO. 7 is G, Table 1, a primer for HNP-1.

SEQUENCE I.D. NO. 8 is H, Table 1, a primer for HNP-1.

SEQUENCE I.D. NO. 9 is I, Table 1, a primer for pre-pro cartridge.

SEQUENCE I.D. NO. 10 is J, Table 1, a primer for pre-pro cartridge.

SEQUENCE I.D. NO. 11 is K, Table 1, a primer for pre-pro cartridge.

SEQUENCE I.D. NO. 12 is L, Table 1, a primer for pre-pro cartridge.

SEQUENCE I.D. NO. 13 is M, Table 1, a primer for *Hind* III, to *Sal*I adaptor.

SEQUENCE I.D. NO. 14 is N, Table 1, a primer for factor X recognition site and an *Sph*I to *Sal*I adaptor.

SEQUENCE I.D. NO. 15 is O, Table 1, a primer for factor X recognition site and an *Sph*I to *Sal*I adaptor.

SEQUENCE I.D. NO. 16 is P, Table I, a primer for an *Eco*RI to *Bam*HI adaptor.

SEQUENCE I.D. NO. 17 is Q, Table I, a primer for a methionine residue and an *Sph*I to *Bam*HI adaptor.

SEQUENCE I.D. NO. 18 is R, Table I, a primer for a methionine residue and an *Sph*I to *Bam*HI adaptor.

SEQUENCE I.D. NO. 19 is S, Table I, a primer for an *Nde*I to *Bam*HI adaptor.

SEQUENCE I.D. NO. 20 is T, Table I, a sequencing primer.

SEQUENCE I.D. NO. 21 is U, Table I, a sequencing primer.

SEQUENCE I.D. NO. 22 is the amino acid sequence of HNP-1.

SEQUENCE I.D. NO. 23 is the amino acid sequence of CEME.

SEQUENCE I.D. NO. 24 is the amino acid sequence of CEMA.

SEQUENCE I.D. NO. 25 is V, Table I, a primer for CEMA.

SEQUENCE I.D. NO. 26 is W, Table I, a primer for CEMA.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: HANCOCK, ROBERT E.W.
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(ii) TITLE OF INVENTION: CATIONIC PEPTIDES AND METHOD FOR PRODUCTION

(iii) NUMBER OF SEQUENCES: 26

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE: 20-AUG-1993
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: WETHERELL, JR., PH.D., JOHN R.
(B) REGISTRATION NUMBER: 31,678
(C) REFERENCE/DOCKET NUMBER: PD2823

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 619/455-5100
(B) TELEFAX: 619/455-5110

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..104

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGGGATCCG CATATGAAAT GGAAACTGTT CAAGAAGATC GGCATCGGCG CCGTGCTGAA 60

AGTGCTGACC ACCGGTCTGC CGGCGCTGAT CAGCTAACTA AGTA 104

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 112 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..112

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCTTACTTA GTTAGCTGAT CAGCGCCGGC AGACCGGTGG TCAGCACTTT CAGCACGGCG 60

CCGATGCCGA TCTTCTTGAA CAGTTTCCAT TTCATATGCG GATCCCCGCA TG 112

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..81

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGAGCTCCT AACTAACTAA GGAGGAGACA TATGAAACAA AGCACTATTG CACTGGCACT 60

CTTACCGTTA CTGTTTACCC C 81

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCAGTGCAAT AGTGCTTTGT TTCATATGTC TCCTCCTTAG TTAGTTAGGA GCTCC 55

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..65

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGTGACAAAA GCCGCATGCT ACTGCCGTAT ACCGGCCTGC ATCGCGGGCG AACGTCGTTA 60

CGGTA 65

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..64

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAGGCCGGTA TACGGCAGTA GCATGCGGCT TTTGTCACAG GGCTAAACAG TAACGGTAAG 60

AGTG 64

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCTGCATCTA CCAGGGCCCT CTGTGGGGGT TCTGCTGCTA AAAGCTTCGC 50

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 76 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..76

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGAAGCTTT TAGCAGCAGA ACCCCCACAG ACCCCCCTGG TAGATGCAGG TACCGTAACG 60

ACGTTCGCCC GCGATG 76

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..110

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCATATGAGG ACCCTCGCCA TCCTTGCTGC CATTCTCCTG GTGGCCCTGC AGGCCCAGGC 60

TGAGCCACTC CAGGCAAGAG CTGATGAGGT TGCAGCAGCC CCGGCAGCAGA 110

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTGCAGCTGA CATCCCAGAA GTGGTTGTTT CCCTTGCATG GGACGAAACG TTGGCTCCAA 60

AGCATCCAGC CTCAAGGAAA AACATGGCAT G 91

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..109

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCATGTTTTT CCTTGAGCCT GGATGCTTTG GAGCCAAGCT TTCGTCCCAT GCAAGGGAAA 60

CAACCACTTC TGGGATGTCA GCTGCAATCT GCTCCGGGGC TGCTGCAAC 109

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..92

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTCATCAGCT CTTGCCTGGA GTGGCTCAGC CTGGGCCTGC AGGGCCAGCA GGAGAATGGC 60

AGCAAGGATG GCGAGGGTCC TCATATGGCA TG 92

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..12

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCTTGTCGA CA 12

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..24

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGTCGACATC GAAGGTCGTG CATG 24

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..24

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CACGACCTTC GATGTCGACG CATG 24

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..12

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AATTCGGATC CG 12

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGGATCCATG GCATG 15

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCATGGATCC GCATG 15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..12

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TATGGGATCC CA 12

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..23

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCAAAATCGG ATCTGATCGA AGG 23

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAGATCGTCA GTCAGTCACG 20

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ala Cys Tyr Cys Arg Ile Pro Ala Cys Ile Ala Gly Glu Arg Arg Tyr
1 5 10 15

Gly Thr Cys Ile Tyr Gln Gly Arg Leu Trp Ala Phe Cys Cys
20 25 30

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..26

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly Ala Val Leu Lys Val
1 5 10 15

Leu Thr Thr Gly Leu Pro Ala Leu Ile Ser
20 25

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: CEMA

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..28

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly Ala Val Leu Lys Val
1 5 10 15

Leu Thr Thr Gly Leu Pro Ala Leu Lys Leu Thr Lys
20 25

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: CEMA variant primer

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..27

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGCGCTGAAG CTAACTAAGT AAGCTTG 27

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: CEMA variant primer

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..31

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AATTCAAGCT TACTTAGTTA GCTTCAGCGC C 31

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

CLAIMS

1. A method of producing a fusion peptide having a cationic peptide portion and an anionic peptide portion comprising;
   a) inserting a DNA sequence encoding the cationic peptide of interest into an expression vector adjacent to the DNA sequence encoding the anionic carrier peptide;
   b) transforming a host cell with the vector of a) and; culturing the transformed host cell;
   c) recovering the fusion peptide.

2. The method of claim 1, further comprising cleaving the anionic peptide portion from the fusion peptide.

3. The method of claim 1, wherein the cationic peptide is from about 5 to about 50 amino acids.

4. The method of claim 3, wherein the cationic peptide contains at least 4 amino acid residues, each with side chains having a net positive charge at pH 7.0.

5. The method of claim 1, wherein the anionic carrier peptide is a pre-pro peptide or an outer membrane protein peptide.

6. The method of claim 1, wherein the anionic carrier peptide is selected from the group consisting of glutathione-S-transferase, protein A and outer membrane protein F.

7. The method of claim 1, wherein the expression vector further contains a spacer DNA sequence between the DNA sequence encoding the anionic carrier peptide and the DNA sequence encoding the cationic peptide.

8. The method of claim 7, wherein the spacer DNA sequence comprises a DNA sequence encoding a proteolytic enzyme recognition site.

9. The method of claim 8, wherein the proteolytic recognition site is recognized by Factor Xa.

10. The method of claim 7, wherein the spacer DNA sequence comprises a DNA sequence encoding an amino acid selected from the group consisting of methionine, tryptophan and glutamic acid.

11. The method of claim 1, wherein the expression vector contains a phenotypic selection marker DNA sequence.

12. The method of claim 11, wherein the phenotypic selection marker is selected from the group consisting of beta-lactamase and chloramphenicol acetyltransferase.

13. The method of claim 1, wherein the expression vector contains a signal sequence.

14. The method of claim 1, wherein transformation is by electroporation.

15. The method of claim 1, wherein the host cell is prokaryotic.

16. The method of claim 15, wherein the prokaryotic cell is selected from the group consisting of *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*.

17. The method of claim 1, wherein the expression of the fusion peptide is directed by an inducible or a constitutive promoter.

18. The method of claim 17, wherein the inducible promoter is the promoter of the *lacZ* gene.

19. The method of claim 17, wherein the constitutive promoter is selected from the group consisting of the promoter of the protein A gene and the promoter of the OprF gene.

20. An isolated peptide, comprising Sequence ID No. 24.

21. An isolated polynucleotide which encodes the peptide of Sequence ID No. 24.

22. A method of inhibiting the growth of bacteria comprising contacting the bacteria with an inhibiting effective amount of the peptide of claim 20.

23. The method of claim 22, wherein the bacteria is gram positive.

24. The method of claim 23, wherein the bacteria is *Staphylococcuus aureus*.

25. The method of claim 22, wherein the bacteria is gram negative.

26. The method of claim 25, wherein the bacteria is selected from the group consisting of *E. coli*, *P. aeruginosa*, *S. typhimurium*, and *E. cloacae*.

27. The method of claim 22, wherein the peptide is administered in combination with at least one antibiotic.

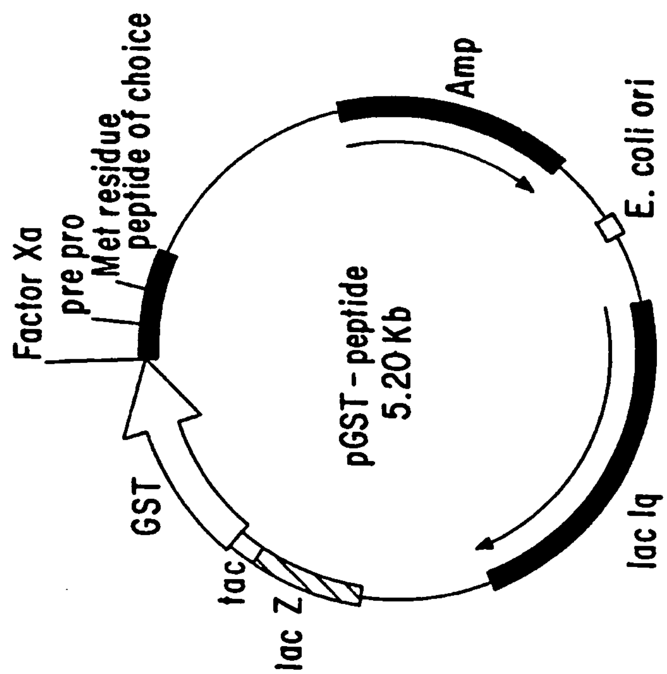
Factor Xa
pre pro
Met residue
peptide of choice
Amp
E. coli ori
pGST-peptide
5.20 Kb
GST
tac
lac Z
lac Iq

FIG. 1A

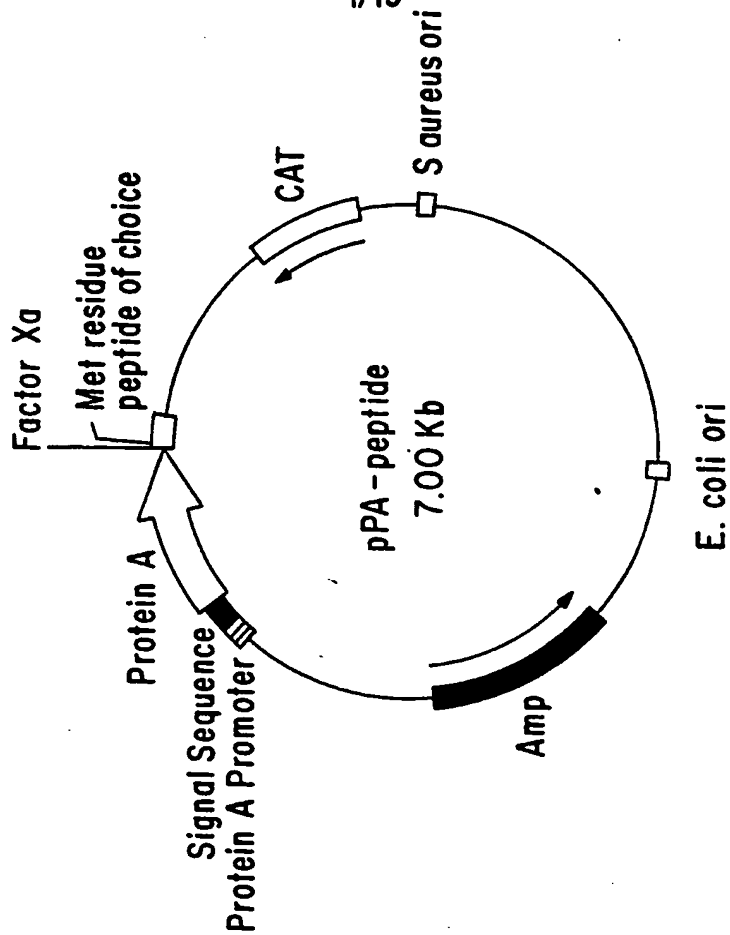
Factor Xa
Met residue
peptide of choice
CAT
S aureus ori
pPA-peptide
7.00 Kb
Protein A
Signal Sequence
Protein A Promoter
Amp
E. coli ori

FIG. 1B

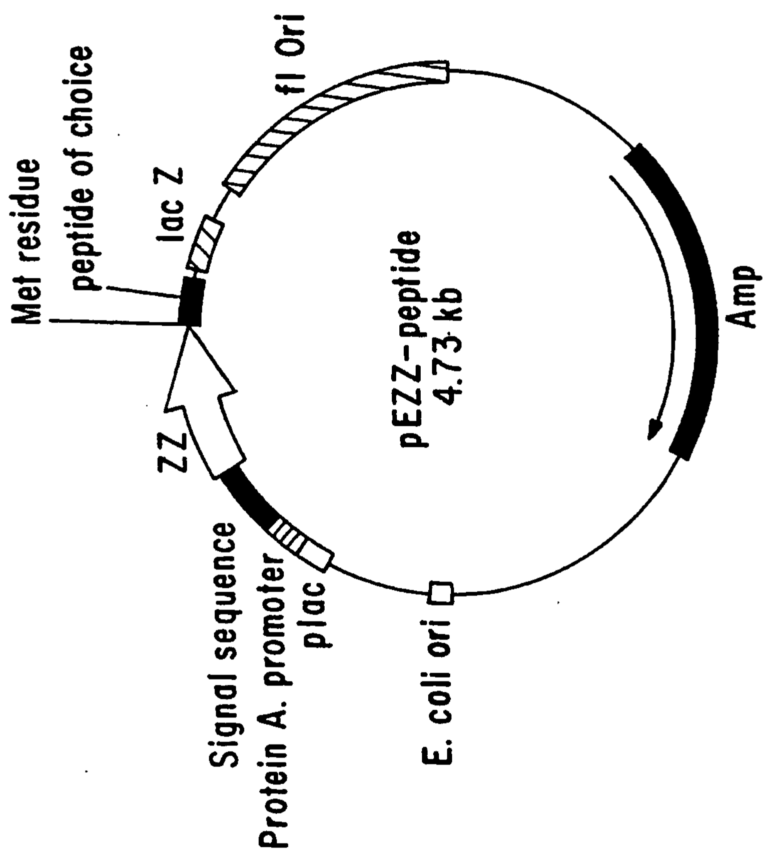
Met residue
peptide of choice
lac Z
fl Ori
Amp
pEZZ-peptide
4.73 kb
ZZ
Signal sequence
Protein A. promoter
plac
E. coli ori

FIG. 1C

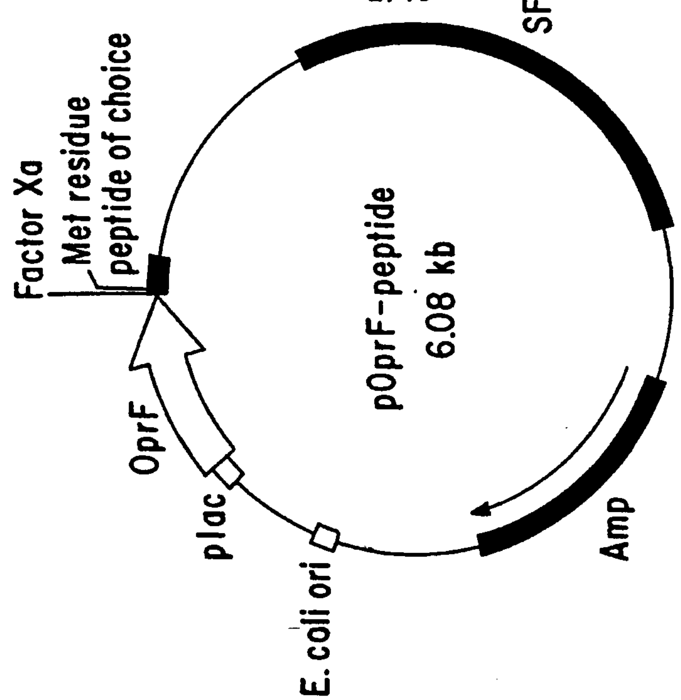
Factor Xa
Met residue
peptide of choice
SF
pOprF-peptide
6.08 kb
OprF
plac
E. coli ori
Amp

FIG. 1D

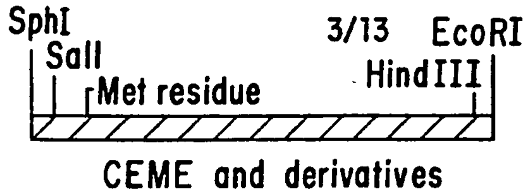

FIG. 2A

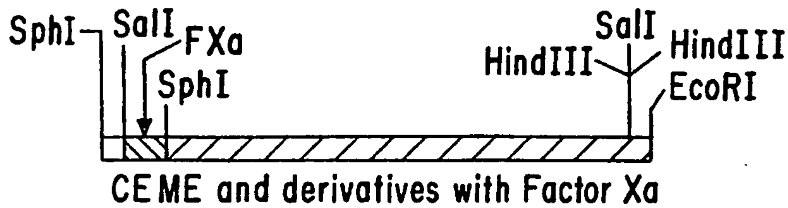

FIG. 2B

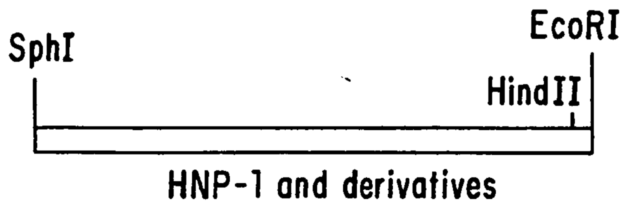

FIG. 2C

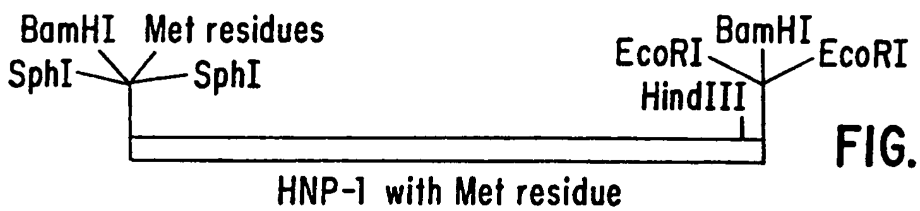

FIG. 2D

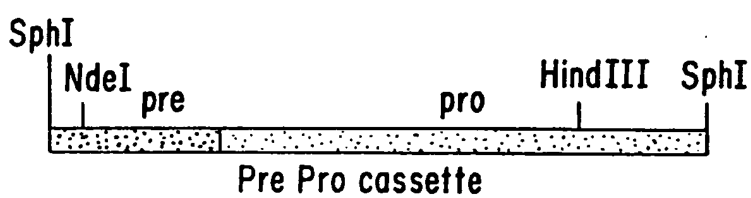

FIG. 2E

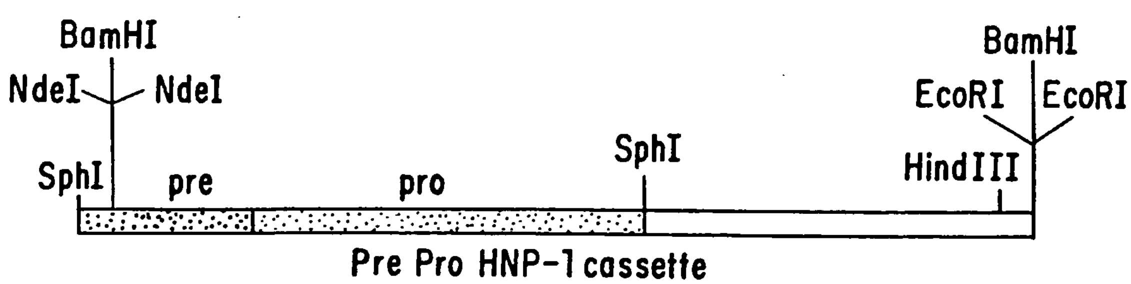

FIG. 2F

HNP-1	A C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
CEME	K W K L F K K I G I G A V L K V L T T G L P A L I S
CEMA	K W K L F K K I G I G A V L K V L T T G L P A L K L T K

FIG. 3

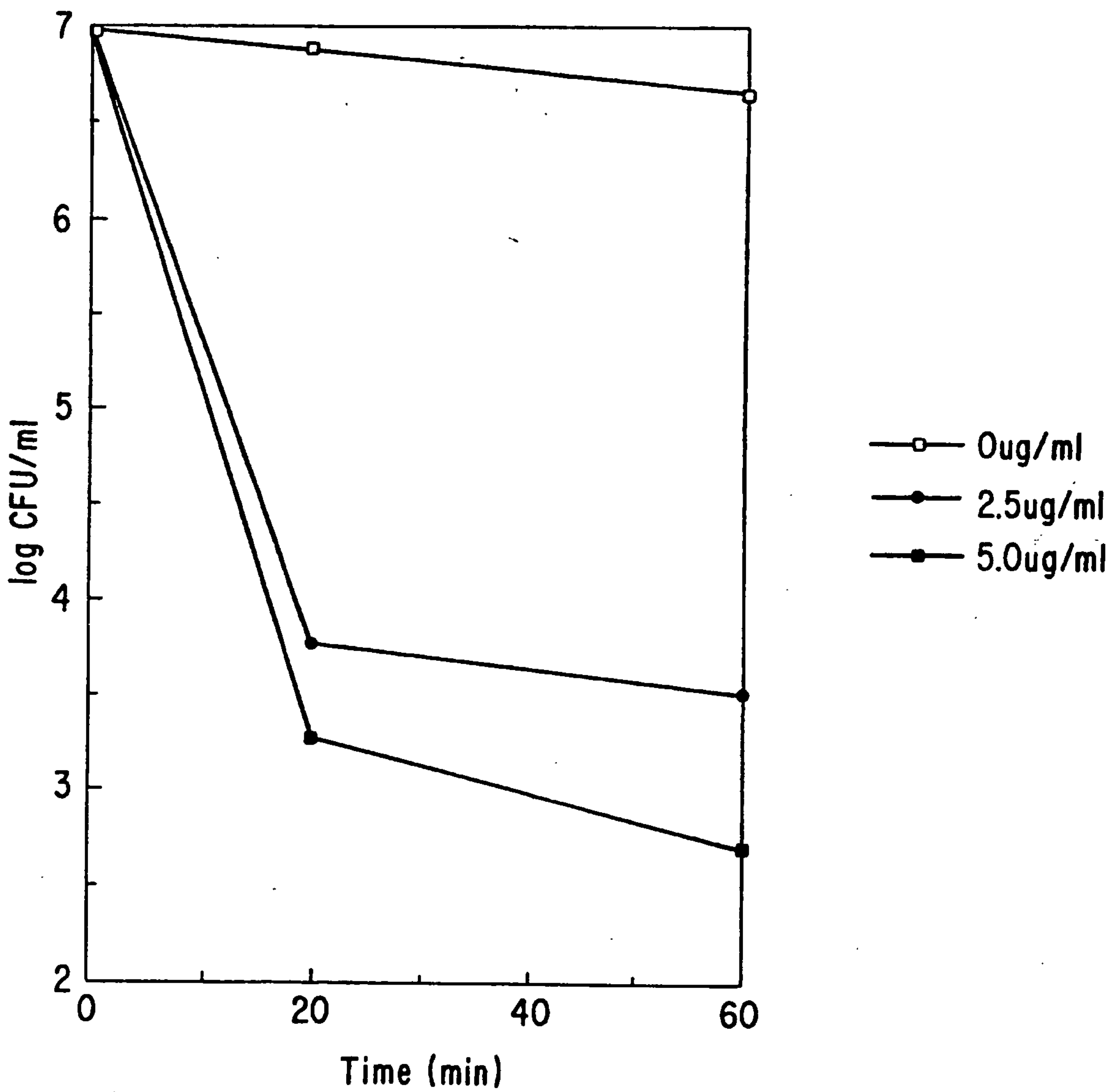

FIG. 4

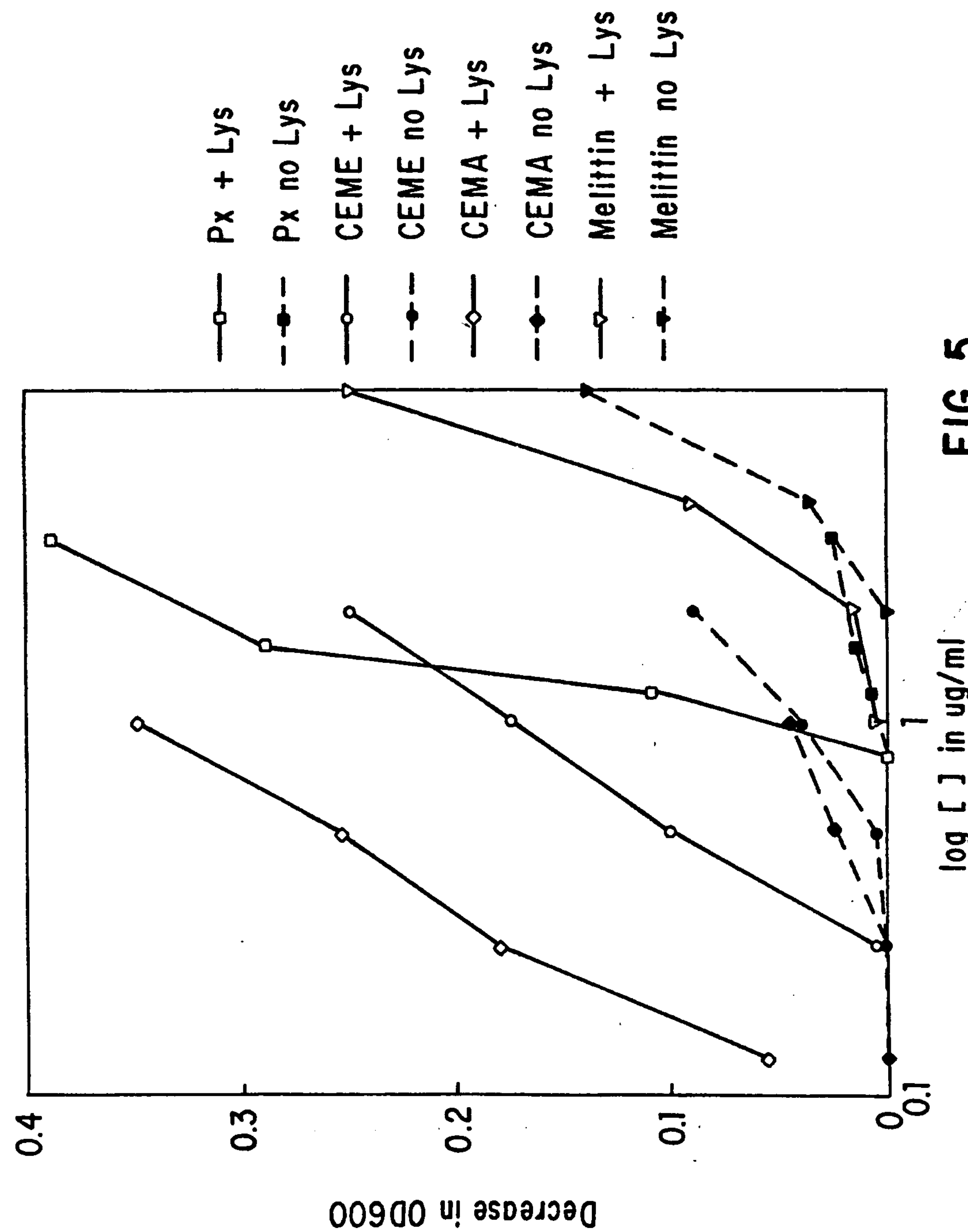

FIG. 5

SUBSTITUTE SHEET

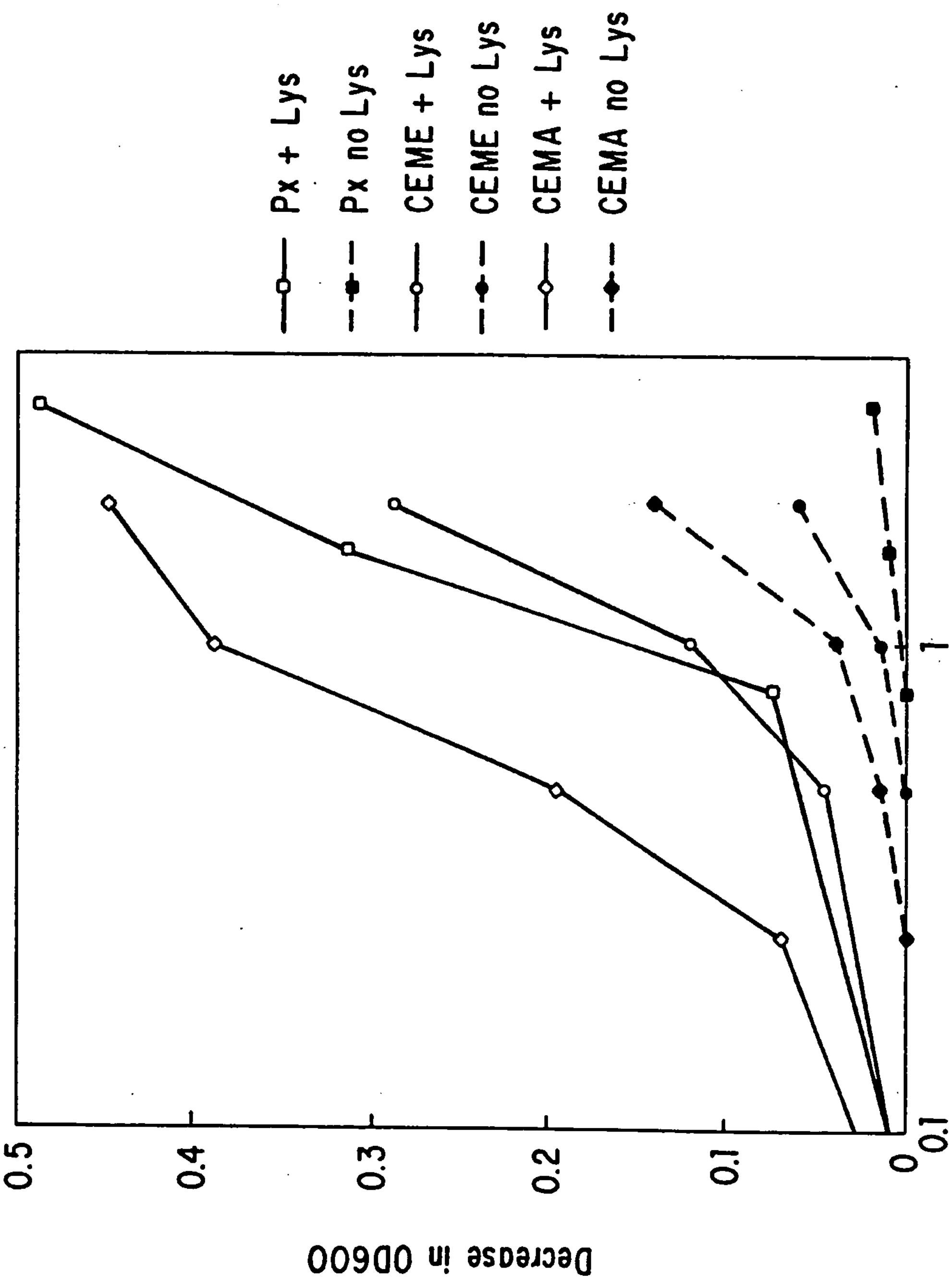
Px + Lys
Px no Lys
CEME + Lys
CEME no Lys
CEMA + Lys
CEMA no Lys
0.5
0.4
0.3
0.2
0.1
0
0.1
1
log [ ] ug/ml
Decrease in OD600

FIG. 6

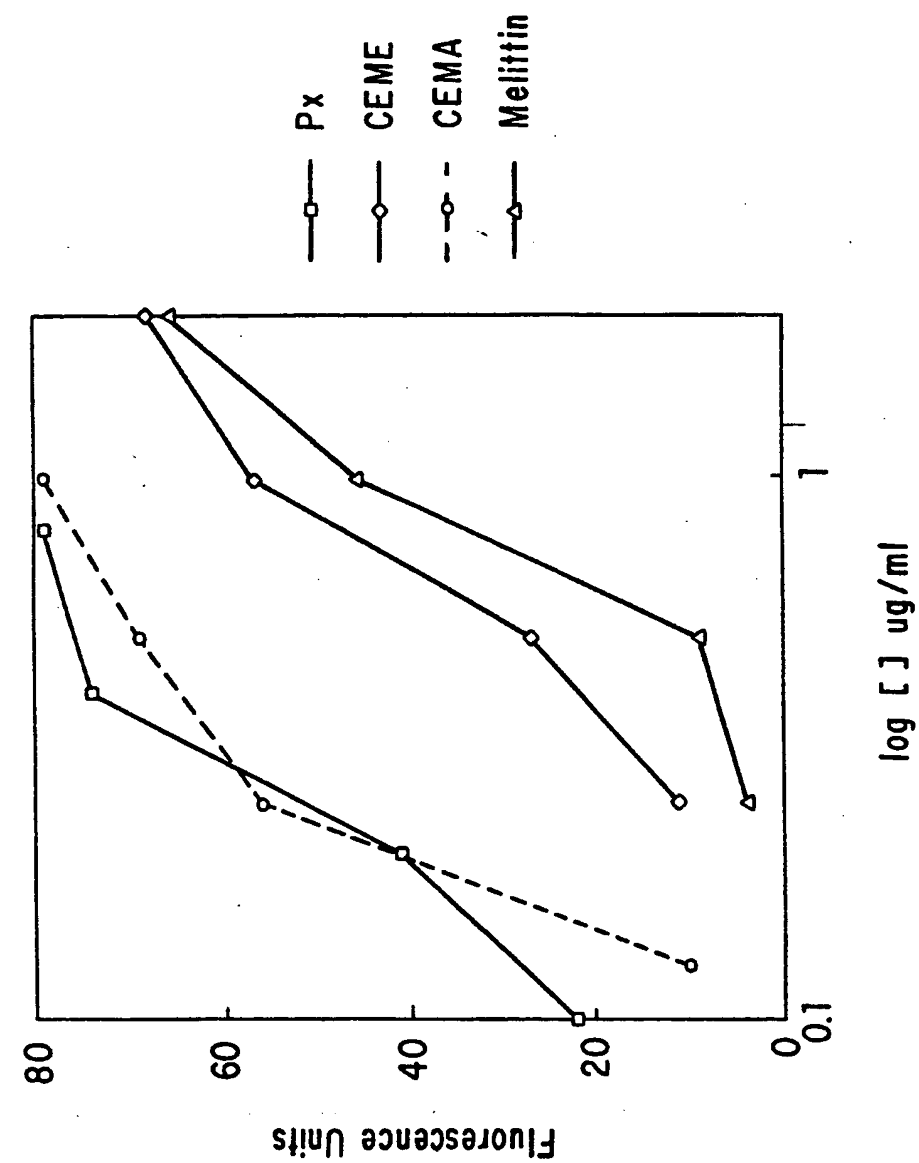
Px
CEME
CEMA
Melittin
Fluorescence Units
80
60
40
20
0
0.1
1
log [ ] ug/ml

FIG. 7

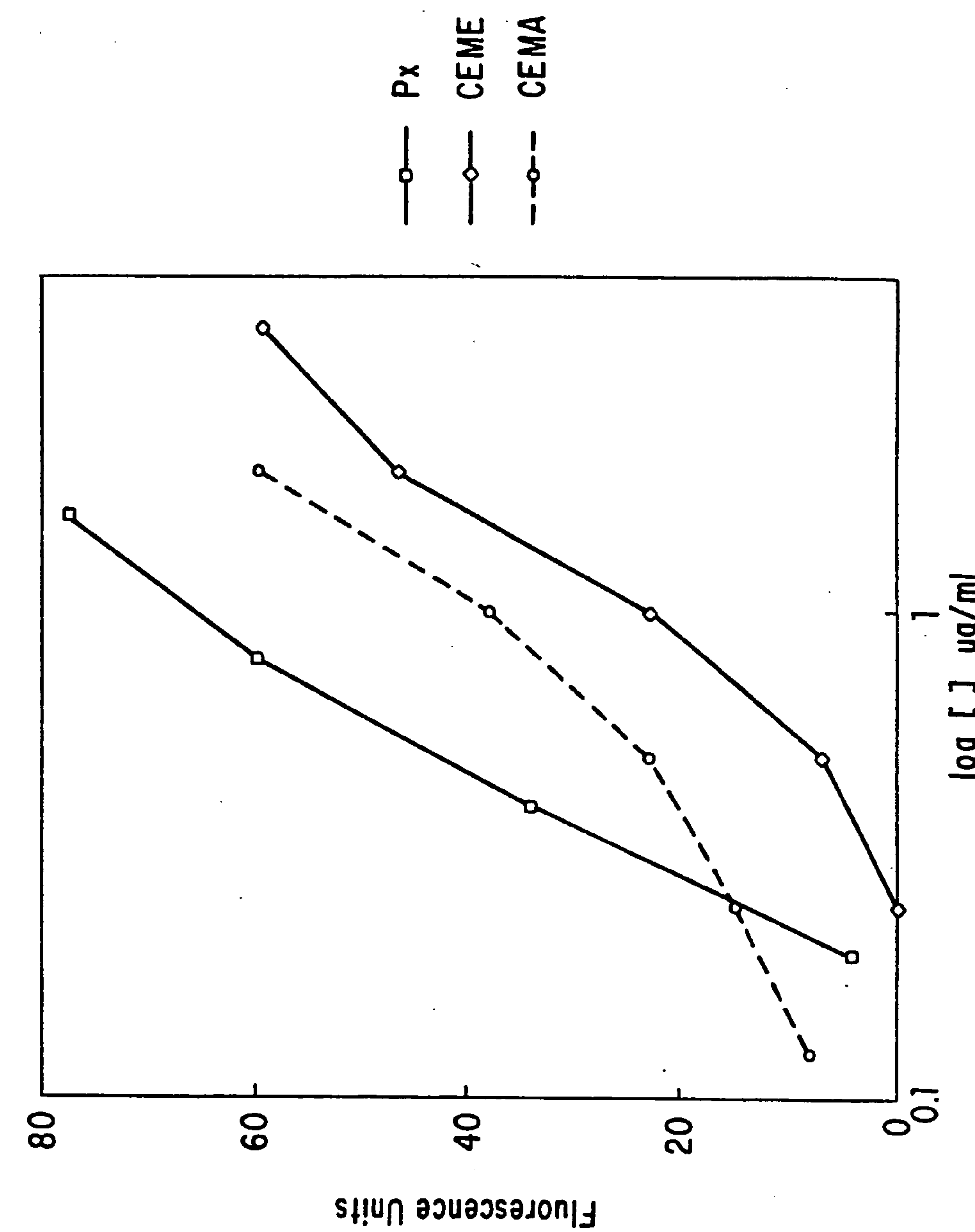

FIG. 8

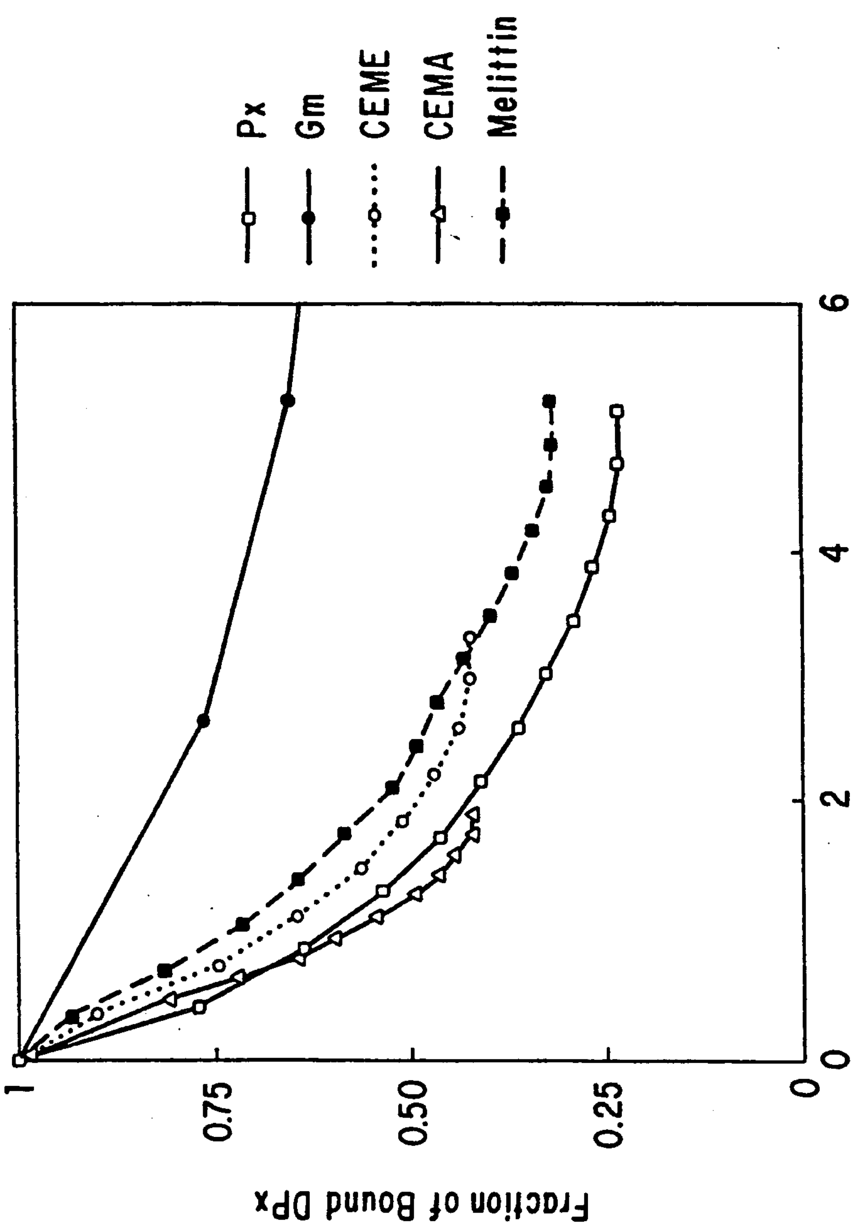
Px
Gm
CEME
CEMA
Melittin
Fraction of Bound DPx
1
0.75
0.50
0.25
0
uM (1)
0
2
4
6

FIG. 9

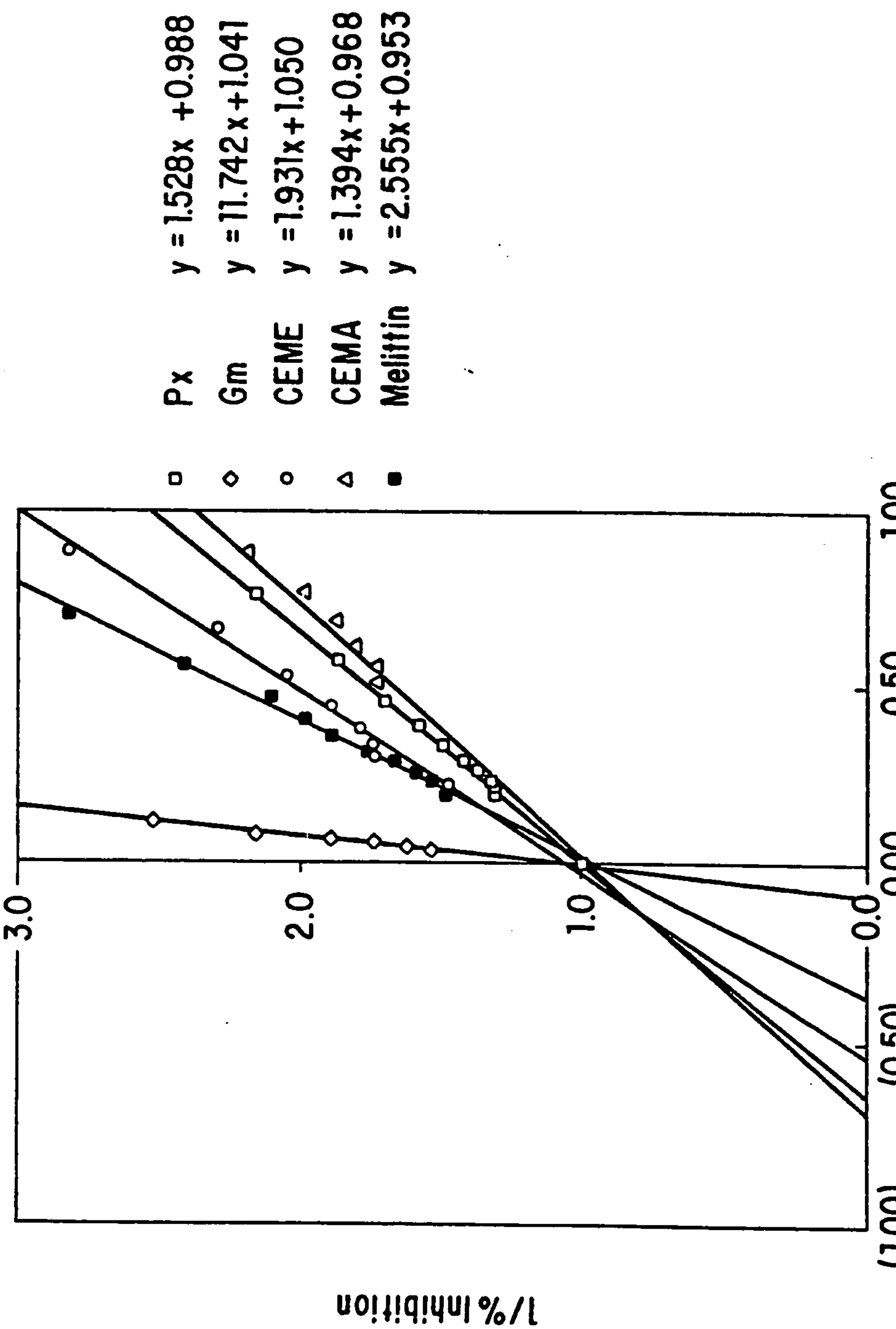

FIG. 10

SUBSTITUTE SHEET

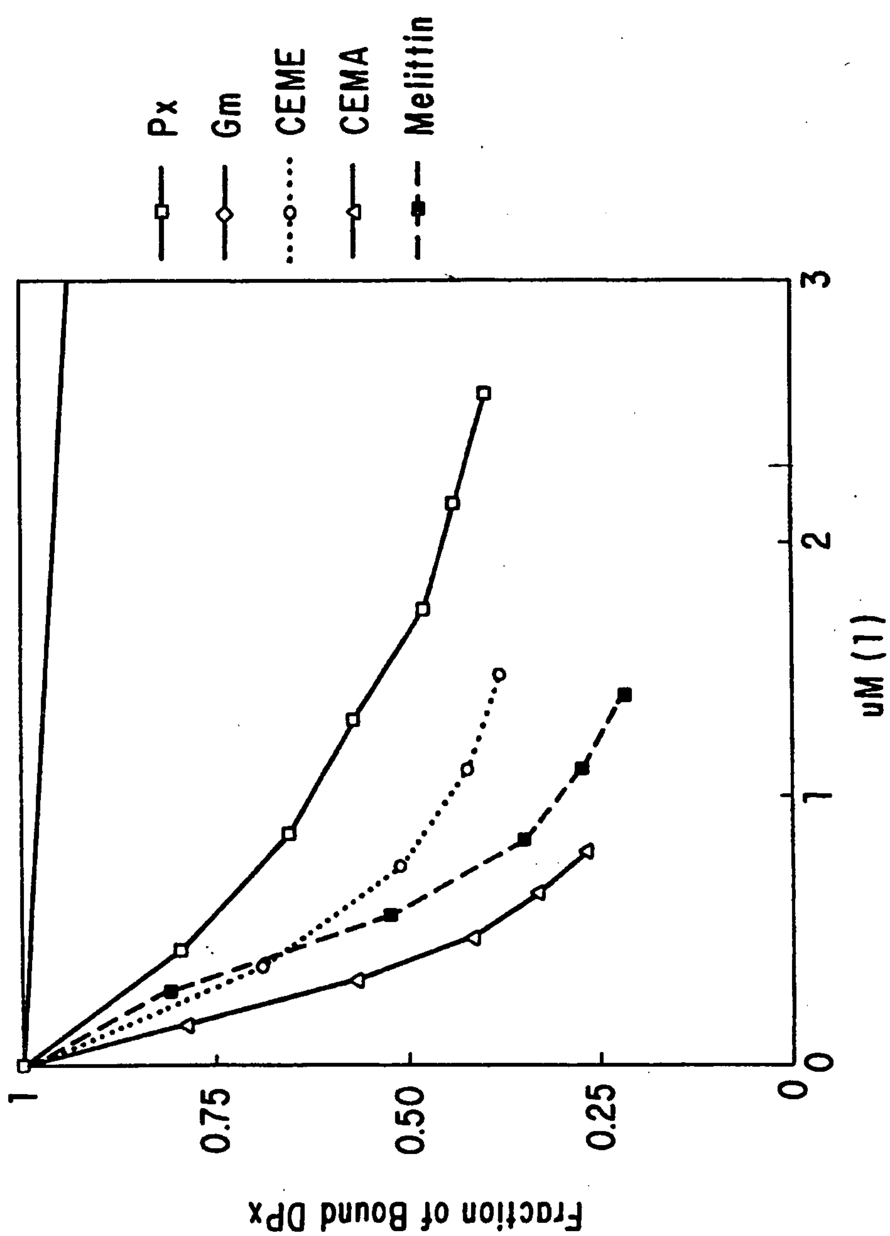
Px
Gm
CEME
CEMA
Melittin
Fraction of Bound DPx
1
0.75
0.50
0.25
0
0
1
2
3
uM (I)

FIG. 11

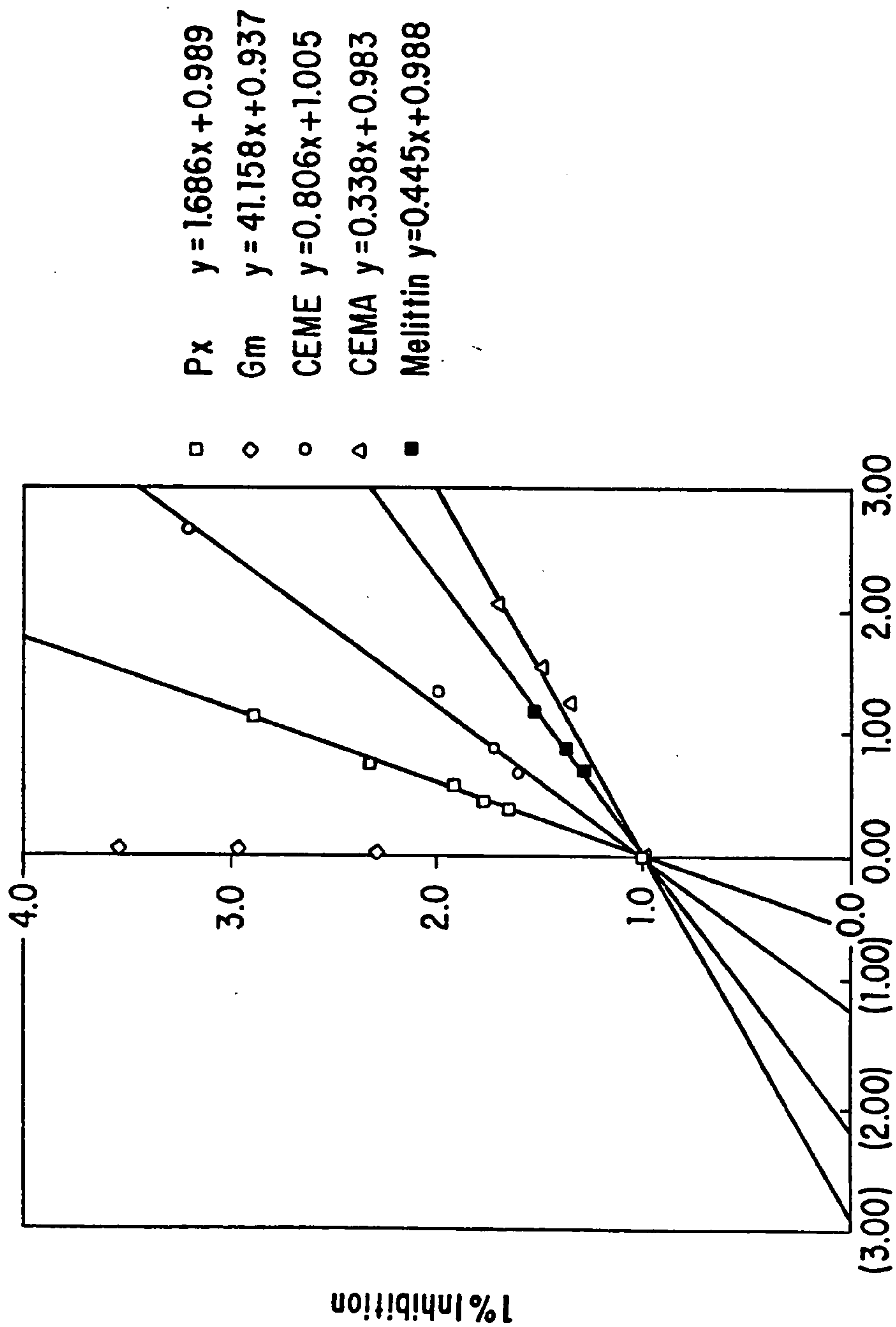
Px y = 1.686x + 0.989
Gm y = 41.158x + 0.937
CEME y = 0.806x + 1.005
CEMA y = 0.338x + 0.983
Melittin y = 0.445x + 0.988
4.0
3.0
2.0
1.0
0.0
(3.00)
(2.00)
(1.00)
0.00
1.00
2.00
3.00
1/I
1% Inhibition

FIG. 12

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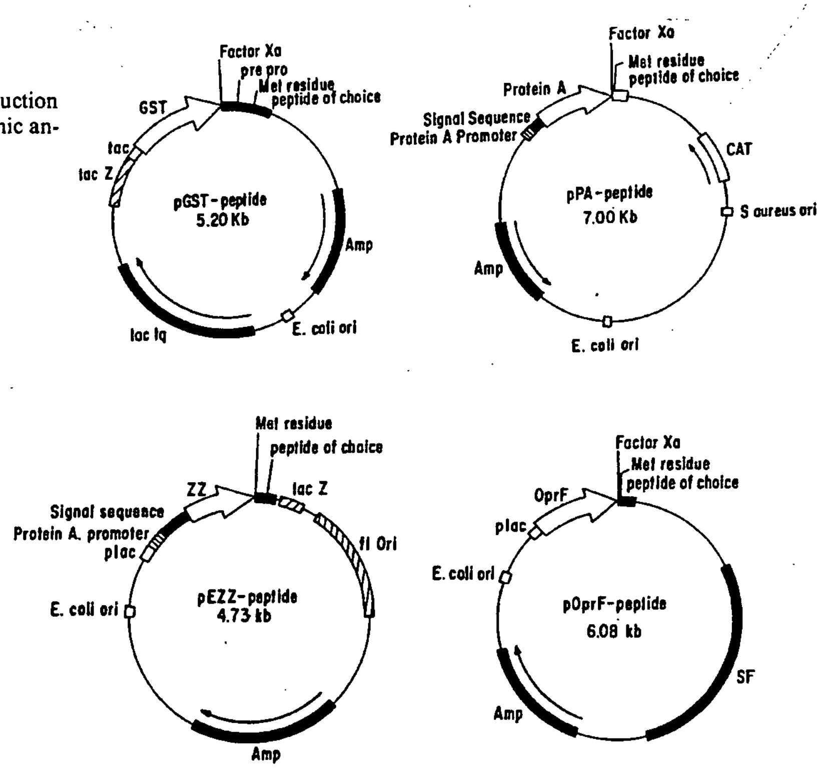
(57) Abstract

Method for recombinant production of cationic peptides and a polycationic antibacterial peptide.



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BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

INTERNATIONAL SEARCH REPORT

Intern. ...al Application No
PCT/CA 93/00342

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/62 C12N15/54 C07K15/08 C07K13/00 A61K37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE BIOCHEMICAL JOURNAL, vol.280, no.1, 15 November 1991 pages 219 - 224 DAIGA ANDERSONS ET AL. 'Biologically active and amidated cecropin produced in a baculovirus expression system from a fusion construct containing the anitody-binding part of protein A' see abstract see page 219, left column, paragraph 1 - right column, paragraph 2 see page 219, right column, last paragraph - page 220, left column, paragraph 3 see page 220, right column, paragraph 4 - page 222, left column, paragraph 1 see page 223, left column, last paragraph - right column, paragraph 1 --- -/--	1-6,13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 1993	30.08.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	MONTERO LOPEZ B.

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 356 409 (KABIGEN AB) 28 February 1990 see column 1, line 1 - line 17 see column 1, line 58 - column 2, line 60 see column 3, line 21 - line 54; examples 1-5 ---	1-6,13
A	WO,A,91 12270 (GENENTECH, INC.) 22 August 1991 see page 5, line 33 - page 6, line 3 see page 7, line 3 - page 8, line 15 see page 15, line 24 - page 17, line 32 see page 18, line 32 - page 20, line 33 -----	1-19

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.-claims 1-19: Method for producing a fusion peptide having a cationic portion and an anionic portion.
2.-claims 20-27: Isolated peptide comprising sequence ID n°24, polynuceotide encoding it and method of inhibiting bacterial growth using such peptide.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-19

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internr   al Application No
PCT/CA 93/00342

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0356409	28-02-90	JP-A- 2135094	23-05-90
WO-A-9112270	22-08-91	US-A- 5227469	13-07-93